Detection of Novel Nucleic Acid Markers in Bodily Fluids

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ABSTRACT

DETECTION OF NOVEL NUCLEIC ACID MARKERS IN BODILY FLUIDS

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The presence of cell-free nucleic acids in bodily fluids of humans has been described for decades. Recent technological advances have opened up numerous possibilities for noninvasive molecular analyses. This thesis aimed at exploring the biology and diagnostic potentials of a novel class of circulating nucleic acid, microRNAs (miRNAs), in the plasma of pregnant women and also of transrenal DNA (Tr-DNA) in the urine of patients undergoing hematopoietic stem cell transplantation (HSCT).

Previously, no data have been published on the presence of miRNAs in human plasma. The first part of this thesis aimed to develop a quantitative method for analyzing cell-free miRNA in plasma. Because miRNAs are very short, with only 21-25 nucleotides, extraction of circulating miRNA was performed using a modified extraction protocol which can efficiently retain the small RNA fraction. Moreover, the quantification of miRNAs was performed using the Taqman® MicroRNA Assays (Applied Biosystems) which employ stem-loop primers to reverse transcribe the short miRNAs into longer cDNA molecules. Using these specifically designed extraction and detection methods, I demonstrated, for the first time, the presence of high concentration of cell-free miRNA in maternal plasma. These findings have laid the foundation for the development of circulating miRNAs as a class of markers for noninvasive monitoring.

The second part of this thesis was aimed at systematically identifying a list of placental miRNAs potentially detectable in maternal plasma during pregnancy. The levels of 157 miRNAs were systematically analyzed. MiRNAs expressed at much higher levels in the placenta than maternal blood cells, but not detectable in maternal plasma after delivery were identified. Using this approach, I identified the placental miRNAs miR-141 and miR-149. The temporal profile of one placental miRNA in maternal plasma across different trimesters was also studied. To further decipher the physical natures of placental miRNAs, maternal plasma was passed through filters and quantified for miRNA. No change in miRNA concentration was observed after filtration. Moreover, naked, purified miRNA molecules showed a slower degradation than purified messenger RNA when incubated in plasma and were still detectable for up to 2 hours. This part of the thesis has unveiled placental miRNA in maternal plasma as a novel class of circulating nucleic acids with potential for noninvasive prenatal monitoring.

In the third part of this thesis, I analyzed cell-free DNA in urine using a sex-mismatched HSCT model. With a newly devised MassEXTEND^{TM} assay accurately quantifying the fractional concentration of donor-derived DNA and quantitative PCR assays studying DNA size distribution, I discovered that a considerable fraction of donor-derived DNA and also another fraction of high-molecular weight DNA originated from cells, probably shed from the urinary tract, in the cell-free urinary DNA of patients undergoing sex-mismatched HSCT. Such findings would enlighten investigators in this field on the further development of better molecular assays targeting these DNA fractions in urine for different noninvasive diagnostic purposes.

The results presented in this thesis have not only increased the current understanding on miRNA in plasma and cell-free DNA in urine, but have also laid the ground work for developing them into noninvasive markers for many potential clinical applications.

摘要

近數十年，人類體液中游離核酸的研究廣泛地出現在各文獻中。而近年生物技術上的進步為非損傷性分子分析提供了更多的可能性。論文宗旨在（一）探討孕婦血漿中游離微型核糖核酸（miRNA）及（二）研究血幹細胞移植病人尿液中穿腎盂脫氧核糖核酸（Tr-DNA）的生物特徵和臨牀醫學應用。

科學界一直未對血漿中是否存在 miRNA 這問題下定論。因此，本篇的第一部份集中於研究定量方法來測量血漿中游離 miRNA 的濃度。因為 miRNA 只由大約 21 至 25 個核甘酸所組成，十分短小，所以游離 miRNA 的抽取技術也一再修改，新的抽取技術能有效地保留較短的 RNA。另外，miRNA 定量分析用了 TaqMan® MicroRNA Assays （Applied Biosystems）的莖環結構引子，這引子能逆轉錄 miRNA 為適當長度的互補脫氧核糖核酸（cDNA），使聚合酶鏈反應能成功地進行。運用這些經特別設計的抽取和定量分析方法，本人首次證實了血漿中存在著大量的游離 miRNA，令此項研究能發展成為非損傷性產前診斷的標誌物奠定了重要的基石。

本篇的第二部份有系統地篩選出一系列出現在孕婦血漿中的游離胎盤 miRNA。經過分析 157 條 miRNA 在不同組織中的濃度，最後篩選出多條胎盤

miRNA，包括 miR-149 和 miR-141，它們在胎盤的濃度比它們在母親血液細胞的濃度高出十倍或以上，並隨著分娩後消失於母親的血漿中。另外，此部份亦分析了血漿中游離胎盤 miRNA 的物理特徵。首先，運用過濾方法，血漿中的游離胎盤 miRNA 的濃度在過濾前後並沒有統計學上的差異。再者，雖然從血漿中被抽取的 miRNA 沒有與微粒狀物質聯繫，但是在血漿中的衰變率仍比信使 RNA 慢，而即使它們在血漿停留兩小時，仍可被測試出來。這些發現，對發展檢測游離胎盤 miRNA 成為非損傷性產前診斷有著重大的意義。

本篇的第三部份利用性別配錯血幹細胞移植病人的尿液作為研究尿液中游離 DNA 的模具。運用 MassEXTEND™ 分析病人尿液中器官捐贈者游離 DNA 的百分濃度，亦運用實時定量鏈反應技術分析病人尿液中游離 DNA 的大小分佈。研究發現，病人尿液中存在相當分量的器官捐贈者的游離 DNA 及血幹細胞移植病人本身的游離 DNA。這些發現，有助發展尿液中游離 DNA 成為非損傷性診斷的重要指標。

綜上所述，本篇的研究不僅使學界對血漿游離 miRNA 和尿液中的游離 DNA 的生物學本質有更透徹的了解，其測量方法更為醫學領域提供了一種嶄新的診斷思路，為將來能發展出的更多不同的非損傷性檢查作出貢獻。

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CHAPTER 1: CELL-FREE NUCLEIC ACIDS IN HUMAN BODILY FLUIDS

1.1 Early studies on the presence of cell-free nucleic acids in human bodily fluids

In as early as the mid 20th century, Mandel and Métais (1948) were the first to measure and compare the levels of nucleic acids in the plasma of patients and healthy persons. This was the first documentation of the existence of cell-free nucleic acids in human bodily fluids. However, few studies continued to pursue on the presence and applications of cell-free nucleic acids in human bodily fluids until the late 1960s, when Tan *et al.* (1966) showed that in patients with systemic lupus erythematosus, there was an elevated level of circulating DNA in serum. In the following decades, the clinical values of circulating nucleic acids gradually emerged. The presence of plasma/serum DNA was demonstrated in rheumatoid arthritis (Koffler *et al.* 1973, Leon *et al.* 1977a), pulmonary embolism (Barada *et al.* 1980) and hemodialysis (Steinman and Ackad 1977).

Besides plasma and serum, cell-free DNA was also reported to be present in other human bodily fluids such as urine (Botezatu *et al.* 2000), amniotic fluid (Bianchi *et al.* 2001), cerebrospinal fluid (Angert *et al.* 2004), and peritoneal fluid (Cioni *et al.* 2003). These findings, together with the numerous technological advances, have triggered a growing tide of interest in the study of cell-free nucleic acids in human bodily fluids as a potential molecular tool for molecular analyses.

1.2 Circulating nucleic acids in plasma and serum

Cell-free circulating nucleic acids in plasma/serum are noninvasive sources of

genetic material for applications in molecular tests. They can be easily accessed with the collection of peripheral blood samples. Potential applications of these genetic materials were also made possible with technologically advances, e.g. real-time quantitative polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR. Together, they have provided important future venues for noninvasive cancer detection and prenatal diagnosis.

1.2.1 Cancer Detection

The detection of cell-free tumor-derived nucleic acids in the circulation provides a convenient and noninvasive approach for cancer diagnosis. Conventional diagnostic and assessment methods for cancer detection have a number of drawbacks. For example, when collecting cells and biopsies for cytological and histological analyses, such as the Papanicolao smear and colonoscopy, the invasive procedures may result in traumatic or discomforting effects on the patients. Other assessment techniques, such as computed tomography (CT) and magnetic resonance imaging, are noninvasive but are so expensive that it limits their applications as screening tests. Thus, there is an increasing need for the development of noninvasive approaches for cancer detection. This is facilitated with the discovery of circulating tumor-derived nucleic acids.

1.2.1.1 Circulating tumor-derived DNA

Early studies demonstrated increased levels of cell-free DNA in plasma/serum of patients with different malignancies (Leon *et al.* 1977b, Shapiro *et al.* 1983, Stroun *et al.* 1987). Of particular interest, the levels of cell-free DNA in the serum of patients with metastases were higher than those without metastases (Leon *et al.* 1977b); treated patients with higher plasma/serum levels of cell-free DNA were associated

with a poor prognosis (Leon *et al.* 1977b). Although these early studies did not address the identity of the tumor-derived DNA, they suggested that circulating cell-free DNA in plasma/serum could play a role in cancer detection and monitoring.

Since then, the development of sequence-specific DNA detection techniques, e.g. the PCR, has further catalyzed the use of cell-free DNA in plasma/serum for cancer detection. Different types of tumor-derived mutated DNA sequences were identified in the plasma/serum of cancer patients. These include, firstly, oncogenes, such as the *Ras* mutation, in the plasma/serum of patients with pancreatic cancer (Mulcahy *et al.* 1998, Yamada *et al.* 1998, Castells *et al.* 1999, Theodor *et al.* 1999), colorectal cancer (Anker *et al.* 1997, de Kok *et al.* 1997, Kopreski *et al.* 1997, Hibi *et al.* 1998), and myelodysplastic syndrome or acute myelogenous leukemia (Vasioukhin *et al.* 1994). Second, mutated sequences of tumor suppressor genes, e.g. mutated *p53* sequence, in patients with hepatocellular carcinoma (Kirk *et al.* 2000) and large bowel cancer (Mayall *et al.* 1998). Third, DNA sequences with microsatellite alterations in patients diagnosed with primary head and neck squamous cell carcinoma (Nawroz *et al.* 1996) and small cell lung cancer (Chen *et al.* 1996). Fourth, hypermethylated promoter DNA sequences in patients with breast cancer (Hoque *et al.* 2006), colorectal cancer (Bazan *et al.* 2006), liver cancer (Wong *et al.* 1999), lymphoma (Deligezer *et al.* 2003), cervical cancer (Yang *et al.* 2004), and melanoma (Marini *et al.* 2006). Finally, DNA sequences of different tumor-associated virus were also found in cancer patients (Mutirangura *et al.* 1998, Gallagher *et al.* 1999, Capone *et al.* 2000, Pornthanakasem *et al.* 2001). The successful detection of the various tumor-derived DNA sequences in the plasma/serum of cancer patients has established the foundation for the noninvasive detection of cancers.

Of significant interest, circulating tumor-derived DNA has several advantages over the more traditional cancer detection methods. For example, plasma DNA markers are potentially applicable for early cancer diagnosis and screening (Eisenberger *et al.* 2006). Subsequent studies have also revealed their potentials in prognostication (Castells *et al.* 1999, Kawakami *et al.* 2000, Lo *et al.* 2000a, Taback *et al.* 2001, Gautschi *et al.* 2004), disease recurrence (Lo *et al.* 1999b, Gonzalgo *et al.* 2002, Ryan *et al.* 2003), and treatment outcome (Lo *et al.* 2000b, Kimura *et al.* 2004, Taback *et al.* 2004). In brief, circulating tumor-derived DNA has enabled a new class of noninvasive markers with unprecedented advantages over the more traditional cancer detection methods.

1.2.1.2 Circulating tumor-derived RNA

Although circulating tumor-derived DNA exhibits overwhelming potentials for developing noninvasive cancer detection methods, researchers in the field have been in constant search for another type of nucleic acid, that is circulating tumor-derived messenger RNA (mRNA), for cancer detection. The detection of circulating tumor-derived mRNA has several advantages in terms of higher sensitivity and specificity. First, each single cell possesses multiple copies of mRNA, as compared with one genome equivalent of DNA copies. Second, expression of mRNA can be tumor-, tissue- or even cell-specific. Therefore, the detection of these mRNA in plasma not only opens up the possibility for highly sensitive noninvasive cancer detection, it may also enable the development of tailor-made approaches to monitoring of cancer progression.

Using the RT-PCR, Kopreski *et al.* (1999) and Lo *et al.* (1999a) were the first to report the presence of tumor-derived mRNA in the serum and plasma of cancer

patients, respectively. In particular, Kopreski *et al.* (1999) showed that the tumor-derived *TYR* mRNA detected in serum was free from contaminating tumor cells, i.e. they are cell-free. The presence of cell-free mRNA in the plasma/serum is surprising given the lability of RNA and the elevated level of serum RNases in cancer patients (Reddi and Holland 1976). Subsequent studies were able to detect, in plasma/serum of cancer patients, candidate mRNA transcripts that are frequently expressed in tumor tissues. They include different telomerase components (Chen *et al.* 2001b, Dasi *et al.* 2001, Miura *et al.* 2003, Novakovic *et al.* 2004), epithelial-expressed genes (Gal *et al.* 2001, Silva *et al.* 2001, Silva *et al.* 2002, Kijima *et al.* 2005), and *beta-catenin* (Wong *et al.* 2004b). In particular, Miura *et al.* (2003) reported that the detection of serum *TERT* mRNA was more sensitive than either α-fetoprotein (*AFP*) or *AFP* mRNA alone in the diagnosis of hepatocellular carcinoma. These results demonstrated some of the advantages of circulating tumor-derived mRNA measurements over conventional diagnostic tumor markers.

In recent years, the use of real-time quantitative RT-PCR has extended the scope and enabled the use of quantitative aberrations in circulating mRNA for cancer detection. Dasi *et al.* (2001) were the first to report that the plasma *TERT* mRNA levels were 7-fold and 10-fold higher in patients with colorectal cancer and follicular lymphoma, respectively, than in healthy controls. In contrast, there were no significant differences in *TERT* mRNA expression in the corresponding cells of the cancer patients and healthy controls. Taken together, these findings reinforced the clinical significance of plasma-based molecular analysis in cancer diagnosis. Subsequent studies also established the diagnostic use of the quantification of circulating tumor-derived mRNA in lung (Sueoka *et al.* 2005, Pelosi *et al.* 2006) and liver cancers (Miura *et al.* 2003).

1.2.2 Prenatal diagnosis

Conventional prenatal diagnoses and assessments have a number of drawbacks. For example, they usually involve the use of invasive techniques such as amniocentesis and chorionic villus sampling. These invasive methods impose a definite risk of miscarriage. In addition, noninvasive prenatal assessment techniques, such as biochemical screening and ultrasound scanning, are associated with a significant false positive rate. Thus, there is a long-standing need for a platform technology for definitive and reliable noninvasive prenatal diagnosis.

Inspired by the "pseudomalignant" nature of the human placenta (Strickland and Richards 1992), researchers demonstrated the existence of circulating cell-free fetal DNA and mRNA in pregnant women as in the release of circulating tumor-derived nucleic acids in cancer patients (Lo *et al.* 1997, Poon *et al.* 2000). Since then, these circulating fetal nucleic acids have provided new possibilities for noninvasive prenatal diagnosis. In addition, the recent discovery of microRNA (miRNA), which has important roles in gene regulation, in placental tissues (Barad *et al.* 2004, Bentwich *et al.* 2005), has raised questions of whether this class of small non-coding RNA can be developed as another novel class of markers.

1.2.2.1 Circulating fetal DNA

Using a Y-chromosome-specific PCR assay, the existence of fetal DNA in maternal plasma/serum was first confirmed in women conceived with male fetuses (Lo *et al.* 1997). In a subsequent study, the same group developed a real-time quantitative PCR assay targeting a gene, the *sex-determining region Y* (*SRY*), on the Y chromosome to determine the fractional concentration of fetal DNA in maternal plasma (Lo *et al.* 1998b). It was found that fetal DNA constituted a mean percentage of 3.4% and 6.2%

in maternal plasma in early and late pregnancies, respectively. Comparing with fetal DNA constitutions, arising from fetal cells, of 0.0035% and 0.008% in the second and third trimesters of pregnancy (Hamada *et al.* 1993), respectively, these results demonstrated the significant enrichment of the extracellular fetal DNA in maternal plasma and the robustness of the detection. Besides, it was found that there was a rapid clearance of circulating fetal DNA after delivery (Lo *et al.* 1999e), which suggested that fetal DNA from previous pregnancies will not persist until subsequent ones. In addition, studies showed that fetal DNA could be reliably detected in maternal plasma/serum from as early as the fifth week of gestation (Honda *et al.* 2002, Rijnders *et al.* 2003). In pregnant women conceived with assisted reproduction, it was shown that fetal DNA appears in maternal plasma from as early as the 18^{th} day after embryo transfer (Guibert *et al.* 2003). These findings demonstrated the feasibility of using circulating fetal DNA for noninvasive prenatal diagnosis from as early as the first trimester.

Fetal gender determination

The initial discovery of circulating Y-chromosome-specific sequences in maternal plasma (Lo *et al.* 1997) inspired the use of the technique for fetal gender determination. Subsequent studies reported that such assessment of fetal gender by plasma DNA is both sensitive and specific (Costa *et al.* 2001, Sekizawa *et al.* 2001a, Wei *et al.* 2001, Zhong *et al.* 2001b). This highly accurate method was useful for risk stratifying families at risk of sex-linked disorders to appropriate clinical management (Costa *et al.* 2002a). This application was proven useful in management of pregnancy at risk of congenital adrenal hyperplasia (Rijnders *et al.* 2001).

Prenatal exclusion of fetal genetic diseases

In maternal plasma, cell-free DNA arises from both maternal and fetal sources. Unlike maternally-inherited fetal DNA which is indistinguishable from the high background of maternal plasma DNA, paternally-inherited fetal DNA is distinguishable from maternal DNA using polymorphic sites, such as mutated or short tandem repeat (STR) sequences. Therefore, most studies on the uses of circulating fetal DNA focused on paternally-inherited fetal DNA.

Detection of mutations in certain autosomal dominant diseases in maternal plasma was a commonly studied application. For example, the detection of expanded CTG trinucleotide repeats in the *dystrophia myotonica protein kinase* gene in maternal plasma was shown to be informative for the prenatal exclusion of myotonic dystrophy (Amicucci *et al.* 2000). Subsequent reports also demonstrated the use of circulating fetal DNA for the prenatal exclusion of autosomal recessive diseases, such as Huntington disease (Gonzalez-Gonzalez *et al.* 2003), cystic fibrosis (Gonzalez-Gonzalez *et al.* 2002), β-thalassemia major (Chiu *et al.* 2002b), and other hemoglobinopathies (Fucharoen *et al.* 2003). However, the application in these cases is more complicated because the mere presence of a recessive allele cannot confirm or exclude the inheritance of the diseases. In light of this problem, Chiu *et al.* (2002a) have demonstrated the feasibility of prenatal exclusion of congenital adrenal hyperplasia using paternally-inherited non-mutant polymorphic markers in fetal DNA.

Several research groups also reported the feasible use of circulating fetal DNA as a tool to monitor fetal aneuploidies. For example, Chen *et al.* (2000, 2001a) demonstrated that STR sequences in circulating fetal DNA can be used for diagnosis

of paternally-inherited fetal aneuploidies. In particular, quantitative aberrations of circulating fetal DNA can also aid the screening of fetal aneuploidies. For example, Lo *et al.* (1999c) and Lee *et al.* (2002a) reported that in pregnant women conceived with fetuses with Down syndrome (trisomy 21), the plasma and serum level of fetal DNA has a 2-fold and 1.7 fold, respectively, increase compared with fetal gender and gestational age matched healthy pregnant women. Moreover, when analyzed in conjunction with the quadruple test from routine biochemical screening, fetal DNA can improve the detection rate from 81% to 86% at a 5% false-positive rate (Farina *et al.* 2003). Increased level of fetal DNA in maternal plasma was also demonstrated in pregnancies affected by trisomy 13 (Wataganara *et al.* 2003). However, there is no screening marker available for the detection of trisomy 13 at present. Moreover, using epigenetic allelic ratio (EAR) analysis of the *maspin* gene, Tong *et al.* (2006) successfully demonstrated the noninvasive prenatal analysis of of trisomy 18.

Indication of fetomaternal well-being

Rhesus D-negative pregnant women with rhesus D-positive fetuses carry an inherent risk of hemolytic disease of the newborn (HDN) as a result of alloimmunization. Faas *et al.* (1998) and Lo *et al.* (1998a) were the first to demonstrated the use of circulating fetal DNA for the prenatal analysis of fetal rhesus D status, which provides information for appropriate clinical management. As HDN is more severe in male fetuses (Ulm *et al.* 1998, Ulm *et al.* 1999), subsequent establishment of multiplex real-time PCR assay by Zhong *et al.* (2001c) for fetal gender and rhesus D status determination further facilitated such a clinical application. Costa *et al.* (2002b) later showed that circulating fetal DNA was highly reliable in rhesus D status determination. Because the noninvasive fetal RHD genotyping is accurate and has

lower associated costs, it has become a noninvasive molecular prenatal diagnostic test to be adopted routinely in the United Kingdom, France, and the Netherlands (Finning *et al.* 2002, Dee *et al.* 2003, Bianchi *et al.* 2005).

Using quantitative real-time PCR assays, several studies reported quantitative aberrations of circulating fetal DNA in the plasma/serum of women with pathological pregnancies, e.g. preterm delivery (Farina *et al.* 2005), polydramnios (Zhong *et al.* 2000), fetomaternal hemorrhage (Lau *et al.* 2000, Samura *et al.* 2003), hyperemesis gravidarum (Sekizawa *et al.* 2001b), and invasive placentation (Sekizawa *et al.* 2002). In particular, in pregnant women with preeclampsia, the levels of circulating fetal DNA were elevated compared with those in normal pregnant women (Lo *et al.* 1999d, Smid *et al.* 2001, Shimada *et al.* 2004). The levels also correlate with disease severity (Zhong *et al.* 2001e) and clinical onset (Leung *et al.* 2001).

1.2.2.2 Circulating fetal messenger RNA

Subsequent to the discovery of circulating tumor-derived mRNA in cancer patients (Kopreski *et al.* 1999), Poon *et al.* (2000) were the first to demonstrate the presence of fetal-specific mRNA in the plasma of pregnant women. The potential use of circulating fetal mRNA for prenatal diagnosis has several advantages over the use of circulating fetal DNA. First, as in tumor-derived mRNA, the much higher copy number of mRNA than genomic DNA per cell may offer higher sensitivity and specificity of detection. Second, the detection and identification of circulating fetal mRNA offer the possibility to overcome the limitation to using gender or genetic polymorphism differences between the mother and the fetus. Moreover, circulating fetal RNA is detectable from as early as the fourth week of gestation (Chiu *et al.* 2006). This suggested the potential of circulating fetal mRNA for early prenatal

analysis. Hence, circulating fetal mRNA offers significant potential for the development of new molecular markers for noninvasive prenatal diagnosis.

Circulating placental mRNAs display a number of physical characteristics. For example, *Ng et al.* (2003b) demonstrated the association of placental mRNAs in plasma with subcellular particles. It was suggested that these placental mRNAs were possibly associated with syncytiotrophoblast microparticles (Gupta *et al.* 2004). Moreover, it was shown that placental mRNAs in the maternal plasma appeared to have a preponderance in the 5' end of the fragments (Wong *et al.* 2005).

Speculation has been placed on the placental origin of circulating fetal mRNA. Poon *et al.* (2000) were the first to demonstrated the presence of fetal-specific Y-linked *zinc finger protein gene* mRNA in maternal plasma. Using real-time quantitative RT-PCR assays, the same group demonstrated the presence of two mRNA transcripts, *human placental lactogen* (*hPL*) mRNA and the beta subunit mRNA of *human chorionic gonadotropin* (*βhCG*) which were specifically expressed in placenta, in maternal plasma (Ng *et al.* 2003b). Subsequent studies were conducted to increase the number of circulating fetal mRNA transcripts available for use in noninvasive prenatal diagnosis. For example, Go *et al.* (2004) screened a panel of potential mRNA transcripts expressed in extraembryonic tissues and demonstrated the detection of placental transcription factor mRNA in maternal circulation. Moreover, Tsui *et al.* (2004) used a systematic and high throughput oligonucleotide microarray approach to identify a number of circulating fetal mRNA transcripts. All of these transcripts showed rapid clearance in maternal circulation which suggested the pregnancy-specificity of their presence and indicated their clinical potentials as noninvasive fetal-specific markers.

Studies were also conducted to demonstrate the association of pathological pregnancies with quantitative aberration of these circulating fetal mRNAs. For example, circulating mRNA transcript of corticotropin-releasing hormone (*CRH*) was shown to be 10-fold elevated in plasma collected from preeclamptic pregnancies when compared with control cases (Ng *et al.* 2003a). Masuzaki *et al.* (2005) suggested that the measurement of plasma mRNA by real-time quantitative RT-PCR can be used as a noninvasive diagnostic, prognostic, and follow-up test for gestational trophoblastic disease. In women carrying trisomy 18 fetuses, the serum concentration of *βhCG* mRNA was 9.4-fold lower than that in normal pregnant women (Ng *et al.* 2004). The presence of chromosome 21-encoded mRNA of placental origin in the plasma of women between the 9^{th} and 13^{th} weeks of pregnancy demonstrated potential for the early prenatal screening of Down syndrome (Oudejans *et al.* 2003). Using mass spectrometry, Lo *et al.* (2007) demonstrated the use of plasma placental RNA ratio in prenatal diagnosis of fetal trisomy 21. Taken together, these studies demonstrated feasibilities of circulating fetal mRNA for noninvasive prenatal diagnosis.

1.2.2.3 Circulating placental microRNA

MicroRNAs (miRNAs) are a family of small (21-25 nucleotides), non-coding RNAs that negatively regulate gene expression at the post-transcriptional level (Ambros 2003, Lai 2003, Bartel 2004). At the time of this writing, no data have been published in relation to the presence of cell-free miRNA in the plasma of humans. Interestingly, Barad *et al.* and Bentwich *et al.* (2004, 2005) have shown the presence of placental-specific miRNAs in human. This has raised the question of whether such small non-coding RNA can indeed be detected in maternal plasma as markers for the

noninvasive prenatal diagnosis as in the detection of placental mRNA.

MiRNAs are associated with various regulatory functions in humans (Bartel 2004). Moreover, they exist at much higher copy numbers in cells than mRNAs (Lim *et al.* 2003). Therefore, they have potential to be developed as highly sensitive prenatal diagnostic markers in maternal plasma. However, considerable challenges have hampered such potential use of miRNA. First, the rich protein content of plasma has posed difficulties for efficient RNA extraction. Second, most column-based protocols for RNA extraction result in great loss of short RNA molecules, which were previously considered as "contaminants" to the longer mRNA molecules. Third, special PCR techniques are required to specifically and sensitively detect these short RNA molecules. Fourth, over 400 human miRNA species have been identified (Griffiths-Jones 2004), but only a minor proportion of them has been studied in detail. Insights into the roles and hence their potential use as diagnostic miRNAs were lacking. It would be one of the major objectives of this thesis to investigating the possibility of developing miRNAs as novel markers for noninvasive prenatal diagnosis. In Chapter 2, detailed description of the biology of microRNA in humans is provided.

1.3 Cell-free nucleic acids in urine

The presence of cell-free DNA in urine offers another novel and completely noninvasive source of genetic materials for clinical analysis. In recent years, same interest has been focused on the study of transrenal DNA (Tr-DNA), which is cell-free DNA in urine derived from circulating cell-free DNA in plasma as it passes through the kidney barrier (Botezatu *et al.* 2000). It is distinct from the high -molecular weight cell-free DNA in urine which is released from cells shed along the

urinary tract. For this latter scenario, passage of DNA through the kidney barrier is not involved. The postulated plasma sources of Tr-DNA suggested its origin from throughout the body, which offers a potentially new diagnostic tool with attractive utility (Umansky and Tomei 2006).

1.3.1 Transrenal DNA (Tr-DNA)

1.3.1.1 Biology of Tr-DNA

Botezatu *et al.* (2000) was the first to demonstrated the existence of Tr-DNA in humans. In brief, this group showed that male-specific sequences could be detected in the urine of women pregnant with male fetuses. Furthermore, *K-ras* mutations associated with colon adenocarcinoma could be detected in the urine of the cancer patients. In addition, Su *et al.* (2004b) showed that Tr-DNA has a length between 150 and 250 bp. They also reported technical refinements, including DNA extraction method and PCR assay design (e.g. amplicon size), which would enhance the detection of such Tr-DNA species (Su *et al.* 2004a).

Several mechanisms through which DNA may be able to pass through the kidney barrier were postulated (Lichtenstein *et al.* 2001, Umansky and Tomei 2006). There were three major hypotheses: (a) Tr-DNA having a size which is below the glomerular filtration cutoff of 65 kDa; (b) the use of large shuntlike pores in the kidney with the involvement of serum amyloid P component on the secondary structure of DNA, and (c) the passage of DNA contained inside liposome-like particles. However, the true mechanism behind remains to be elucidated.

1.3.1.2 Detection of fetal-derived Tr-DNA

Several groups have investigated the possibility of detecting transrenal fetal DNA.

As in plasma where circulating maternally-derived fetal DNA is indistinguishable from maternal cell-free DNA, detection of fetal Tr-DNA depends on gender and genetic DNA polymorphism for its distinction from maternal cell-free DNA in urine, e.g. Y chromosome sequences of male fetuses. Using Y chromosome specific PCR, the initial detection of fetal derived Tr-DNA was reported by Botezatu *et al.* (2000). Subsequent studies reported that the detection of fetal DNA in urine can be achieved from as early as the seventh week of gestation (Al-Yatama *et al.* 2001). These findings suggested that urine Tr-DNA is a novel class of nucleic acids potentially applicable for noninvasive prenatal diagnosis in as early as the first trimester. Using quantitative real-time PCR assays, Koide *et al.* (2005) have shown that fetal-derived Tr-DNA was more readily detectable with PCR amplicon of less than 107 bp. This has provided further technical consideration for the molecular analysis of Tr-DNA.

However, not all workers in the field are convinced with the reliability of detecting fetal-derived Tr-DNA. For example, Zhong *et al.* (2001a) reported inability to detect male-specific DNA in urine samples obtained from women pregnant with male fetuses. In particular, in a cohort of pregnant women suffering from pre-eclampsia, the same group was again unable to detect such fetal-derived Tr-DNA (Li *et al.* 2003). This is especially remarkable because pregnant women with pre-eclampsia have elevated levels of circulating fetal DNA (Zhong *et al.* 2001d) and increased renal permeability, as evidenced by proteinuria (Hayashi *et al.* 2002).

1.3.1.3 Potential problems associated with the detection of Tr-DNA

In addition to the fact that Tr-DNA was reported to be detectable by some but not all investigators in this field, considerable challenges have hampered such potential use of Tr-DNA. First, there is currently no consensus method for the processing,

extraction and detection of cell-free DNA from urine. As a result, fair comparisons of results and conclusion cannot be produced between research groups. Moreover, an accurate analytical system for quantifying the fractional concentration of Tr-DNA in urine is lacking. In turn, such a lack of accurate quantification methods has also impaired the development of better urine DNA extraction protocols. Therefore, it is one of the major objectives of this thesis to evaluate the possibility of Tr-DNA analysis in a sex-mismatched hematopoietic stem cell transplantation (HSCT) system. This model has a higher fractional concentration of donor-derived DNA in plasma than fetal DNA has in maternal plasma. Thus, more donor-derived DNA is expected to pass into the urine and may allow more robust detection. The implementation of this HSCT model has the potential to greatly improve current understanding of the phenomenon of Tr-DNA. It is hopeful that the use of this HSCT model has several advantages over other models being used previously. This fractional concentration of donor-derived DNA in HSCT recipients (Lui *et al.* 2002) is much higher than the mean fractional concentration of fetal DNA in maternal plasma, which is only 3.4% during weeks 11 to 17 of gestation and 6.2% in the third trimester of pregnancy (Lo *et al.* 1998b), and less variable than that of tumor in the plasma of cancer patients (Anker *et al.* 1997). Thus, these results suggested that sex-mismatched HSCT may be a more powerful model system for the investigation on Tr-DNA because donor-derived DNA might be more readily detected in the urine of the HSCT recipients than in other transrenal models.

1.3.2 Cell-free DNA in urine as released from the urinary tract

Cell-free DNA released from the urinary tract has two main channels to enter the urine. First, cells shed from the urinary tract may contribute to the high-molecular

weight cell-free DNA in urine upon cell death. Second, DNA enters the circulation as circulating cell-free DNA which then appears as Tr-DNA. In normal individuals, not much can be done to distinguish the two. However, these two scenarios may be distinguished in special clinical scenarios.

As a demonstration of the shedding of urinary tract DNA into urine, Eisenberger *et al.* (1999) reported the detection of tumor-derived DNA in the urine of renal cancer patients. Tumor-derived DNA was also detectable in the urine and corresponding plasma/serum of patients suffering from prostate carcinoma (Goessl *et al.* 2000, Jeronimo *et al.* 2002) and bladder cancer (Utting *et al.* 2001).

As another example of the shedding of urinary tract DNA into urine, using real-time quantitative PCR assay for Y-chromosome sequences, donor-derived male DNA was also detectable in the urine of female recipients of renal transplants (Zhang *et al.* 1999). Elevated level of urinary DNA was observed during an acute rejection episode. Subsequent to anti-rejection treatment, it showed that the DNA level rapidly returned to a normal level (Zhang *et al.* 1999). This illustrated the potential use of cell-free DNA in urine for organ transplant monitoring.

1.4 Other bodily fluids with cell-free nucleic acids

Cell-free DNA and RNA were also detected in bodily fuilds other than plasma, serum and urine. They include amniotic fluid, cerebrospinal fluid, and peritoneal fluid. This section gives a brief summary of recent progresses in this field. However, it should be noted that the sampling of these bodily fluids involves invasive procedure, they are not subjects for the development of noninvasive tests.

1.4.1 Amniotic fluid

The presence of cell-free fetal DNA in amniotic fluid (AF) was first demonstrated by Bianchi *et al.* (2001). It was found that the concentration of fetal DNA in AF was approximately 100- to 200-fold more than that in maternal plasma. However, unlike cell-free DNA in maternal plasma whose origin is from the placenta, Lun *et al.* (2007) showed the non-placental origin of cell-free fetal DNA in AF using an epigenetic approach.

The large quantity of amniotic cell-free fetal DNA has prompted researcher to explore its diagnostic potentials. For example, Larrabee *et al.* (2004) demonstrated the use of amniotic cell-free fetal DNA in the diagnosis of fetal gender and aneuploidy (Down and Turner syndrome) using comparative genomic hybridization (CGH) analysis. In addition, Lapaire *et al.* (2007) showed that the unique fragmentation signatures of amniotic cell-free fetal DNA are potentially indicative of different fetal aneuploidies (Lapaire *et al.* 2007).

The presence of cell-free fetal mRNA in AF was first demonstrated by Larrabee *et al.* (2005). However, in AF, the level of cell-free fetal mRNA appears to be much lower than that of cell-free fetal DNA (Larrabee *et al.* 2005). Moreover, these mRNAs are not expressed in the placenta (Larrabee *et al.* 2005), and thus may not be useful for diagnosing conditions involving placental pathology, e.g. pre-eclampsia and intra-uterine growth restriction. Although such cell-free nucleic acids may have value for prenatal diagnosis, it should be bear in mind that the collection of AF for such testes is an invasive procedure and impose a definite risk of miscarriage.

1.4.2 Cerebrospinal fluid (CSF)

In pregnant women receiving spinal anesthesia for cervical cerclage, cesarean delivery or postpartum tubal ligation, Angert *et al.* (2004) were the first to demonstrate the presence of cell-free fetal DNA in CSF. In particular, the detection of fetal DNA in CSF postpartum demonstrated a different clearance profile from that in the plasma/serum (Lo *et al.* 1999e). Although the detection showed a limited sensitivity, with only four out of 26 cases with detectable fetal DNA, this study illustrated the passage of circulating cell-free fetal DNA across the blood brain barrier.

1.4.3 Peritoneal fluid

In a case study, Cioni *et al.* (2003) reported the presence of male cell-free fetal DNA in the peritoneal fluid of a pregnant woman with a male fetus. Further studies are required to confirm the findings and to precisely define the origin of these sequences.

CHAPTER 2: MICRORNA IN HUMANS

2.1 Introduction

MicroRNAs (miRNAs) are a family of small (21-25 nucleotides), non-coding RNAs that negatively regulate gene expression at the post-transcriptional level (Ambros 2003, Lai 2003, Bartel 2004). In the early 1990s, the first miRNA, *lin-4*, was discovered in *C. elegans* as small temporal RNA (stRNA) which regulates developmental transition (Lee *et al.* 1993). Studies on this novel class of RNAs remained quiescent until 2000, when *let-7*, another stRNA in *C.elegans*, was discovered (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee and Ambros 2001). Since then, the term "miRNA" was coined as recognition to the importance of such small RNAs with important regulatory functions. Hundreds of miRNAs have been discovered across different species, with diverse expression patterns and regulation of various functions, such as developmental and physiological processes.

In the human genome, it has been estimated that there are over 800 miRNAs (Bentwich *et al.* 2005). They regulate expression of about 30% of the protein coding genes (Lewis *et al.* 2005, Xie *et al.* 2005). Over 450 miRNAs have been documented to-date (Griffiths-Jones 2004). Intensive cloning and computational approaches continue to reveal additional miRNAs. Exponential growth in the field has added a new dimension to our understanding of the complex gene regulatory networks in different biological processes.

2.2 Biogenesis

2.2.1 Transcription of microRNA genes

MiRNAs are encoded as genes. Most of them are expressed as independent

transcription units (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee and Ambros 2001). In addition, some miRNAs are located in the introns of pre-mRNAs (Aravin *et al.* 2003, Lagos-Quintana *et al.* 2003, Lai *et al.* 2003). This arrangement allows the coordinated expression of a miRNA and a protein (Aravin *et al.* 2003). Of particular interest, some other miRNAs are clustered in the genome. It implys transcription of miRNAs as a multi-cistronic primary transcript (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001) sharing functional relationships.

The expression of miRNAs shows distinct profiles in different tissue/cell types and at different time. It is speculated that this property allows ample opportunity for "micromanaging" the output of the transcriptome (Bartel 2004). In addition, expression of miRNAs can result in an abundant number in cells (Lim *et al.* 2003), reaching as many as 50,000 mature molecules per cell. Such findings implied either the more active transcription of miRNAs or an unexpected stability associated with miRNAs.

The genes of miRNAs are first transcribed into a miRNA precursor, primary-microRNA (pri-miRNA), before subsequent processing into biologically active and mature forms. Intron-residing miRNA genes share the same promoter elements and pre-mRNA transcripts with their host protein-coding genes (Lagos-Quintana *et al.* 2003). On the other hand, independent miRNA genes and clustered miRNA genes are transcribed from their own promoters into their own pri-miRNAs (Lagos-Quintana *et al.* 2003). In all cases, transcription of miRNAs is processed by RNA polymerases pol II (Bartel 2004). In addition, Chen *et al.* (2004) showed that there is no obligate link between the identity of the polymerase involved and the downstream miRNA processing and function.

2.2.2 Processing and maturation of microRNA precursors

Pri-miRNAs are very long nascent miRNA transcripts with over 1,000 nucleotides. Subsequent processing is needed before they can turn into biologically active mature miRNAs, which are ~21-25 nucleotides long.

Drosha cleavage of pri-miRNA

Inside the nucleus, the nascent pri-miRNA, which is over 1,000 nucleotides long, folds within itself to form a number of stem-loop structures. It is cleaved by Drosha RNase III endonucleases at sites near the base of the primary stem loop to release a ~60-70 nucleotides long stem-loop miRNA precursor, the pre-miRNA (Lee *et al.* 2002b, Zeng and Cullen 2003). This Drosha cleavage has specificity in defining the ends and the identity of the mature miRNA, probably through recognition of secondary structures and element flanking the primary stem loop (Lee *et al.* 2003). The resulting pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin-5 (Yi *et al.* 2003, Lund *et al.* 2004).

Dicer cleavage of pre-miRNA

In the cytoplasm, Dicer RNase III endonucleases recognize and cleave at a double-stranded stem next to the base of the stem-loop pre-miRNA (Lee *et al.* 2003). The cleavage releases an imperfect duplex of the mature miRNA, ~21-25 nucleotides long, and a similar-sized fragment derived from the opposing arm of the pre-miRNA, the miRNA* (Lau *et al.* 2001). This miRNA:miRNA* duplex is expected to be short-lived, as suggested by the 100-fold less cloning frequency of the miRNA* than that of the miRNA strand (Lagos-Quintana *et al.* 2002, Aravin *et al.* 2003, Lim *et al.* 2003).

RNA-induced silencing complex (RISC) Assembly

Subsequent processing of the miRNA:miRNA* processing resembles the posttranscriptional gene silencing pathway of RNAi and involves the ribonucleoprotein complex (miRNP) (Mourelatos *et al.* 2002), which is a kind of RISC. Upon loading onto the RISC, miRNA* of the miRNA:miRNA* duplex detaches and degrades. The resulting RISC-miRNA complex will conduct sequence specific posttranscriptional silencing activities.

2.3 Mechanisms of gene regulation

The exact mechanism behind miRNA mediated gene regulation remains unknown. In humans, the two most commonly recognized mechanisms are cleavage of mRNA and translational repression. In general, if the miRNA has sufficient complementarity to the mRNA, cleavage of mRNA happens. On the contrary, if there is insufficient complementarity to be cleaved but does have a suitable constellation of miRNA complementary sites, productive translation of the mRNA is repressed (Hutvagner and Zamore 2002, Zeng *et al.* 2002, Doench *et al.* 2003, Zeng *et al.* 2003).

2.3.1 Cleavage of target mRNA

If the RISC-miRNA complex binds with enough complementarity to the target mRNA, it induces cleavage between the nucleotides on the mRNA paired to residues 10 and 11 of the miRNA (Kasschau *et al.* 2003, Palatnik *et al.* 2003). The site of cleavage does not change even if the miRNA is not perfectly paired to the target at the 5' terminus, and it can be located along the whole mRNA. After mRNA cleavage, the RISC-miRNA complex remains intact for cleavage of other mRNAs (Hutvagner and Zamore 2002, Tang *et al.* 2003).

2.3.2 Translational repression of mRNA

In general, miRNAs in metazoan cells regulate gene regulation by translational repression, mainly because of the less extent of complementarity between miRNAs and mRNAs in metazoans (Rhoades *et al.* 2002). The complementary site of miRNAs resides on the 3' UTR of the target mRNAs (Reinhart *et al.* 2000, Brennecke *et al.* 2003, Zeng and Cullen 2003). This mechanism of posttranscriptional regulation is featured with a drop in target protein level without a change in the target mRNA level (Wightman *et al.* 1993). In particular, the combined action of multiple miRNAs on a single mRNA target was shown to have the most efficient translational repression (Lee *et al.* 1993, Wightman *et al.* 1993, Reinhart *et al.* 2000, Abrahante *et al.* 2003, Doench *et al.* 2003, Lin *et al.* 2003). However, the exact repression mechanism, whether it is because of captured translational initiation or stalled ribosome at post-initiation stages (Olsen and Ambros 1999), remains to be elucidated.

2.4 Functional roles of microRNAs

MiRNAs play important roles in various biological processes in humans and other vertebrates. Since miRNAs are usually highly conserved between different organisms, studies conducted in different model organisms provide important understanding of the functional roles of miRNAs in human. Other insights on miRNAs are also accumulating from studies in primary malignant tissue and cancer cell lines.

2.4.1 Oncogenesis

MiRNAs play important roles in the pathogenesis of cancer in human (Chen 2005). Some microRNA genes are located in regions of translocation or deletion characteristic to certain cancers. Subsequent loss of regulatory function results in

cancer. For example, the miR-15a-miR16-1 cluster located on chromosome 13q14 is commonly deleted in chronic lymphocytic leukemia (CLL) (Calin *et al.* 2002). Point mutation of the miR-16-1 precursor also leads to CLL (Calin *et al.* 2005). Moreover, some miRNAs are regulators of commonly recognized oncogenes and tumor-suppressor genes. Altered expressions of these miRNAs result in oncogenic gene expression. For example, in some human lung cancers, reduced *let-7* expression results in increased oncogenes, *RAS*, expression (Johnson *et al.* 2005). In addition, miRNAs can act as oncogenes or tumor-suppressors themselves. For example, in human B-cell lymphomas, the highly expressed miR-17-92 cluster can augment the oncogenic effect the c-*Myc* gene (He *et al.* 2005). Given the specificity of gene regulation by miRNAs, it was suggested that miRNA expression profiling can classify cancers more efficiently than mRNA expression profiling (Lu *et al.* 2005). Thus, miRNAs can potentially be used to develop more efficient methods of cancer diagnosis and treatment. In particular, target prediction of cancer-related miRNAs can potentially enhance the discovery on a new class of tumor suppressors and oncogenes as target for diagnosis and therapy.

2.4.2 Programmed cell death

The association of miRNA with oncogenesis is probably a result of the role of miRNA in regulating programmed cell death (apoptosis). Up to date, most studies of the regulation of miRNA on programmed cell death in humans are related to cancer. For example, in glioblastoma, a highly malignant human brain tumor, miR-21 is strongly expressed. Chan *et al.* (2005) demonstrated the inhibitory effect of miR-21 on apoptosis with measurement of the *caspase-3* and *caspase-7* enzymatic activity, and terminal deoxynucleotidyl-mediated dUTP nicked labeling staining. In addition, miR-16-1 and miR-15a were found to be negatively repressing the expression of

Bcl2, a proapoptotic protein (Cimmino *et al.* 2005). Reduced expression of the two miRNAs inhibits apoptosis and results in CLL.

2.4.3 Cellular differentiation and development

Complex networks of biomolecular interaction are involved during the development of animals. Using different model organisms, studies showed that miRNAs are essential for the normal development of animals.

During early stages of human embryonic stem (ES) cell development, a specific set of miRNAs are downregulated upon differentiation of human ES cells into embryoid bodies (Suh *et al.* 2004). These observations suggested that specific profiles of miRNAs expression are needed for the correct differentiation of the zygote into different cell types. The importance of miRNA was further demonstrated in the zebrafish and mouse with mutant *Dicer*, whose ES cells could not produce mature miRNA that failed to differentiate (Bernstein *et al.* 2003, Wienholds *et al.* 2003, Kanellopoulou *et al.* 2005).

The tissue-specificity pattern of miRNA expression during embryonic developments suggested the importance of miRNAs in the differentiation of different cell/tissue types (Wienholds and Plasterk 2005). A number of studies also reported the developmental functions of different miRNAs. For example, miR-1 controls cardiomyocytes differentiation and development in mice (Zhao *et al.* 2005); miR-181 directs hematopoietic lineage differentiation in B-lymphocytes of mouse bone marrow and the thymus (Chen *et al.* 2004); miR-143 promotes adipocyte differentiation or functioning (Esau *et al.* 2004). In particular, Seitz *et al.* (2003, 2004) reported the association of miR-127 and miR-136 with imprinting during normal development in humans.

2.4.4 Regulation of physiological and cellular processes

Besides being involved in oncogenesis and cellular development, several miRNAs are involved with various physiological and cellular processes in different organisms. For example, miR-375 expressed in murine pancreatic islets cells regulates glucose-stimulated insulin exocytosis (Poy *et al.* 2004). MiR-122a, differentially expressed in mouse testis during spermatogenesis, was reported to be involved in chromatin remodeling during spermatogenesis (Yu *et al.* 2005).

2.5 Aim of this thesis

The presence of cell-free nucleic acids in different bodily fluids in humans has enabled various noninvasive diagnostic tests. This thesis aims at investigating the biological characteristics and diagnostic implications of another two novel classes of cell-free nucleic acids in human bodily fluids.

In the second section of this thesis, I studied circulating miRNA in pregnant women. The existence and quantitative detection of miRNA in human plasma have not been reported in the literature at the time of this writing. I aimed to address the various challenges which might hamper the development of miRNA in plasma as diagnostic markers. In Chapter 4, methods for quantification of miRNA was developed and evaluated in detail. The feasibility of plasma miRNA detection was also addressed. In Chapter 5, a list of candidate pregnancy-specific miRNA markers was developed systematically using the quantitative methods described. Besides, various biological characteristics of cell-free miRNA in plasma were described.

Cell-free DNA in urine has attracted much attention for its development into a tool for noninvasive diagnosis. However, there is no consensus on a method for

quantitative analysis of cell-free DNA from urine. Moreover, an accurate analytical system for quantifying the proportion of DNA that passes through the kidney barrier into the urine is lacking. In the third section of this thesis, in Chapter 6, I studied the possibility of Tr-DNA analysis in hematopoietic stem cell transplantation recipients.

Finally, in Chapter 7, a general conclusion of the studies and the future perspectives of cell-free nucleic acids in human bodily fluids as diagnostic tools are presented.

SECTION II: MATERIALS AND METHODS

CHAPTER 3: QUANTITATIVE ANALYSIS OF CIRCULATING AND URINARY NUCLEIC ACIDS

3.1 Preparation of samples

3.1.1 Preparation of plasma

Peripheral blood was collected into ethylenediaminetetraacetic acid (EDTA)-containing blood tubes. EDTA can work as an anticoagulant by chelating the calcium molecules in blood since calcium is needed for coagulation to occur. Plasma was harvested by double-centrifugation of the blood to minimize blood cell contamination (Chiu *et al.* 2001). The blood samples were first centrifuged at 1600 *g* for 10 min at 4℃ (Centrifuge 5810R, Eppendorf, Germany). Plasma samples were carefully transferred into plain polypropylene tubes and re-centrifuged again at 16,000 *g* for 10 min at 4℃ (Centrifuge 5415R, Eppendorf, Germany). The "cell-free" supernatants were collected into a fresh polypropylene tube.

For DNA studies, plain plasma was stored at -20℃ until DNA extraction. For RNA studies, 1 mL of TRIzol LS Reagent (Invitrogen, CA, USA) was mixed thoroughly with 800 μL of plasma before storage at -80℃. The storage of plasma with TRIzol LS (Invitrogen) is critical for preservation of RNA integrity. Wong *et al.* (2004a) have recently demonstrated the superior integrity of RNA in plasma that had been stored with TRIzol (Invitrogen) for three years, comparing with the same plasma samples which had been stored alone without the TRIzol LS (Invitrogen) (Wong *et al.* 2004a).

3.1.2 Preparation of blood cells

Peripheral blood was collected into EDTA-containing tubes and subjected to centrifugation at 1,600 *g* for 10 min at 4°C (Centrifuge 5810R, Eppendorf, Germany). Buffy coat is the fraction of a centrifuged blood sample that contains most of the white blood cells and platelets, settling between the plasma (top layer) and the red blood cells (bottom layer). After centrifugation, the buffy coat was carefully transferred into fresh polypropylene tubes and re-centrifuged again at 2,300 *g* for 5 min at room temperature (Centrifuge 5415R, Eppendorf, Germany). The upper residual plasma was removed to obtain the lower blood cell layer.

For DNA studies, the blood cell fraction was stored at -20℃ until DNA extraction. For RNA studies, 900 μL of TRIzol LS Reagent (Invitrogen) was mixed thoroughly with 300 μL of the blood cell fraction before storage at -80℃.

3.1.3 Preparation of placental tissue

Placental tissues were obtained from pregnant women immediately after elective cesarean delivery. They were stored in RNAlater™ solution (Ambion®, Austin, TX) according to manufacturer's instructions immediately upon collection. The samples were stored at –80°C until RNA extraction.

3.1.4 Preparation of urine and urine cell pellet

Spontaneous urine specimens were collected into sterile plain bottles. The specimen was mixed with 0.5 mol/L EDTA (Invitrogen, Carlsbad, CA), pH 8.0, to a final concentration of 10 mmol/L to inhibit possible nuclease activities (Milde *et al.* 1999, Botezatu *et al.* 2000). To separate the cell-free and cellular portion of urine, total

urine was firstly centrifuged at 3000 *g* at 4°C for 10 min. The supernatants were then filtered through a 0.45 μm filter (Milex-GV; Millipore, Billerica, MA) to remove any remaining cells or cell debris. Filtered urine was considered cell-free and was then stored at –80 °C until DNA extraction. The urinary cell pellet following centrifugation was washed with 1X phosphate buffered saline (Invitrogen, Carlsbad, CA) twice before storage at –20 °C for subsequent DNA extraction.

3.2 Nucleic acid extraction

3.2.1 Extraction of small RNA-containing total RNA from plasma, blood cells and placental tissue

Plasma and blood cells RNA was isolated by a method modified from the procedure used for plasma and buffy coat RNA isolation (Ng *et al.* 2002, Ng *et al.* 2003b). It was a combination of the use of TRIzol LS Reagent (Invitrogen) and the *mir*Vana™ miRNA isolation kit (Ambion®, Austin, TX), Figure 3.1. Based on the single-step RNA isolation method developed by Chomczynski and Sacchi (1987), TRIzol LS Reagent (Invitrogen) is a monophasic solution of phenol and guanidine isothiocyanate, which is capable of denaturing proteins, including ribonucleases, thus preserving the integrity of ribonucleic acids. Because plasma is a protein-rich bodily fluid, the TRIzol LS Reagent (Invitrogen) was used for the complete denaturation of protein so that more RNA could be released from its binding cellular proteins. The procedure also prevents protein coagulates from clogging the downstream extraction column. The *mir*Vana™ miRNA Isolation Kit (Ambion) is a column-based RNA isolation technology. Unlike other RNA extraction columns which were unable to retain very small RNA sized below 200 nucleotides, the filter cartridge (Ambion) was specially designed for purification of small RNAs, inculding miRNA, which are

about 21-25 nucleotides long. It involves an organic extraction followed by immobilization of RNA on glass-fiber filters to purify small RNA-containing total RNA using a microspin protocol. This approach does not involve the use of cesium chloride gradient ultracentrifugation or alcohol precipitation, which is relatively time-consuming and labor-intensive, therefore providing a fast and simple alternative for the preparation of RNA from blood samples. Placental tissue RNA was isolated by a method similar to that in plasma and blood cells. However, TRIzol Reagent (Invitrogen) was used instead of TRIzol LS Reagent (Invitrogen) for solid tissue RNA extraction.

For the isolation of total RNA from plasma, 1.6 mL of each sample was mixed with 2 mL of TRIzol LS Reagent (Invitrogen) and 0.4 mL of chloroform. For the isolation of total RNA from blood cells, 0.3 mL of each sample was mixed with 0.9 mL of TRIzol LS Reagent (Invitrogen) and 0.24 mL of chloroform. For isolation of total RNA from placental tissues, 50-100 mg of placental tissue was homogenized in 1.5 mL TRIzol Reagent (Invitrogen), and 0.3 mL chloroform was added. Cell-debris was removed by centrifugation at 12,000 *g* for 15 min at 4°C (Centrifuge 5415R; Eppendorf). To 1.5 mL of homogenate, 0.4 mL of chloroform was added and mixed. The mixtures were separated into different phases by centrifugation at 12,000g for 15 min at 4°C (Centrifuge 5415R; Eppendorf, Germany): RNA in the aqueous phase, DNA in the interphase, and proteins at the organic phase. The aqueous layer was carefully removed and transferred into fresh polypropylene tubes.

Total RNA in the aqueous layer was then extracted using the *mir*Vana™ miRNA Isolation Kit (Ambion) according to the manufacturer's protocols. First, for adjusting the filter cartridge binding conditions, 1.25 volumes of absolute ethanol were added

to one volume of the aqueous layer. The mixture was applied to *mir*Vana™ filter cartridges (Ambion) followed by centrifugation at 10,000 *g* for 10 sec (Centrifuge 5415D; Eppendorf), during which total RNA was adsorbed to the membrane. After all mixture was passed through the filter cartridge, 700 μL of miRNA Wash Solution 1 (proprietary to the manufacturer) was loaded onto the filter cartridge to wash away contaminants by further spins under the same conditions. Afterwards, the filter cartridge was subjected to another 500 μL of miRNA Wash solution 2/3 (proprietary to the manufacturer). High-speed centrifugation at 10,000 *g* for 1 min (Centrifuge 5415D; Eppendorf) was then performed for the complete removal of residual buffer from the membrane. Lastly, to elute RNA from the filter cartridge, 100 μL of pre-heated RNase-free water (at 95°C) was added directly onto the membrane and incubated at room temperature for 1 min, followed by centrifugation at 10,000 *g* for 1 min (Centrifuge 5415D; Eppendorf).

At this stage, to ensure isolation of pure RNA from the samples, DNase I digestion was performed using the Amplification Grade DNase I (Invitrogen, Carlsbad, CA). After elution from the *mir*Vana™ filter cartridge (Ambion), each RNA sample was quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). For digestion, the following four components were added to fresh microcentrifuge tubes on ice: 1 μg of RNA sample, 1 μL of DNase I, 1 μL of 10X Reaction Buffer, and RNase-free water to 10 μL. This reaction mixture was incubated at room temperature for 15 min, followed by the addition of 1 μL of 25 mM EDTA solution and incubation at 65°C for 10 min for to inactivate DNase I. The treated RNA samples were then stored at -80°C prior to analysis within 2 weeks.

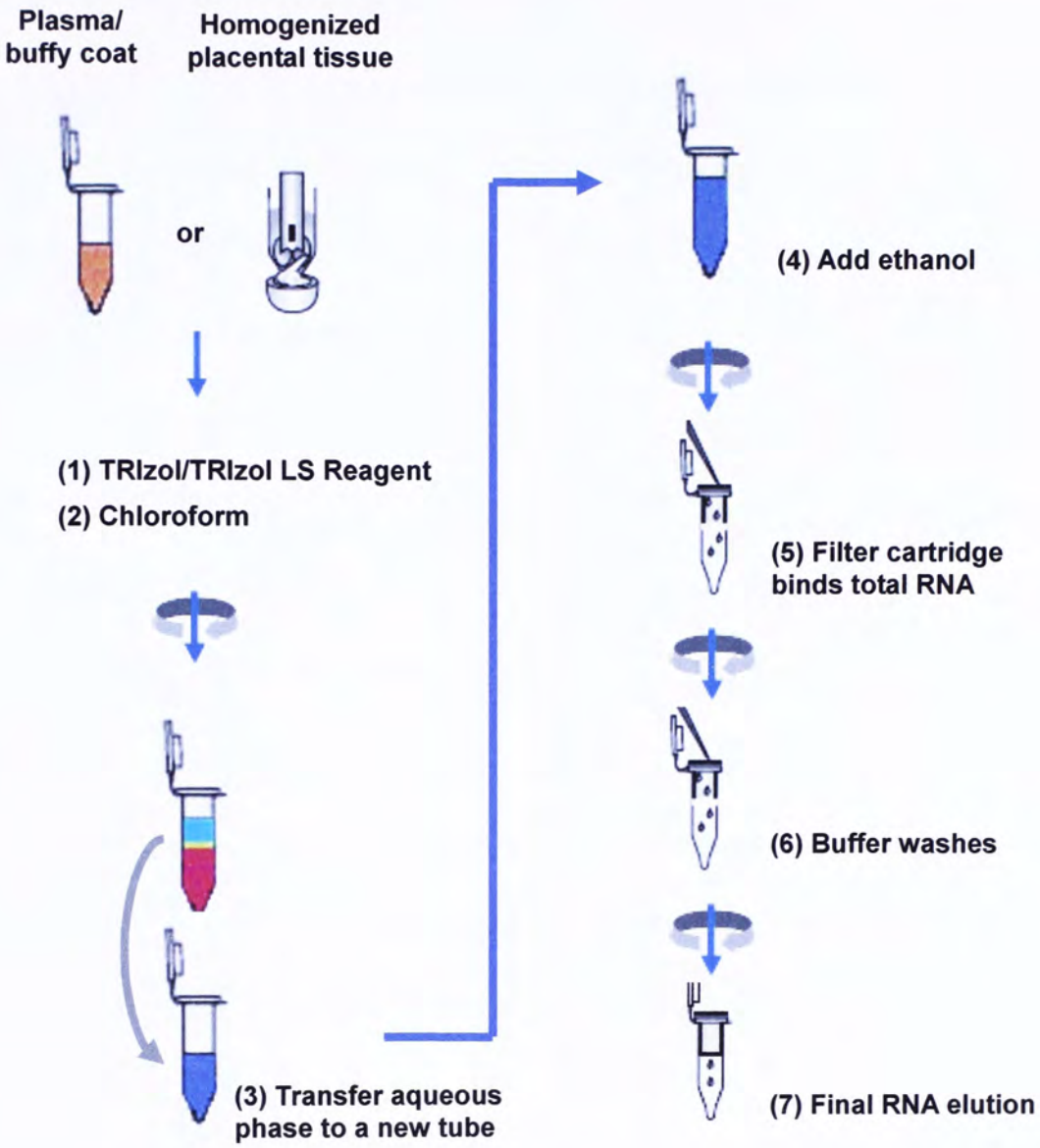


Figure 3.1 Procedure for RNA extraction from plasma, blood cells and placental tissue. (Modified from *mir*Vana™ miRNA Isolation instruction manual, Ambion, 2006)

3.2.2 Extraction of DNA from plasma, blood cells and urine cell pellet

DNA was extracted from plasma, blood cells and urine cell pellet using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the 'blood and bodily fluid spin protocol' (Lo *et al.* 1998b). For plasma DNA extraction, 800 μL of plasma was used. For blood cell DNA extraction, 300 μL of buffy coat was used. For urine cell pellet DNA extraction, urine cell pellet was first resuspended in 200 μL of 1X phosphate buffered saline (Invitrogen) before DNA extraction. For each 400 μL of fluid sample, 40 μL of QIAGEN Protease and 400 μL of AL buffer were added and the mixture was incubated at 56℃ for 10 min. After that, 400 μL of cold absolute ethanol was added. The mixture was transferred onto a QIAamp Spin Column and centrifuged at 16,000 *g* for 1 min. The extraction column was washed twice with AW1 and AW2 buffers, at 16,000 *g* for 1 min and 3 min, respectively. DNA was finally eluted in 50 μL of deionized water, by centrifugation at 16,000 *g* for 1 min. The extracted DNA was stored at -20℃ until analysis.

3.2.2 Extraction of DNA from urine

For extraction of DNA from the filtered cell-free urine, 15 mL of 6 mol/L guanidine thiocyanate (Sigma-Aldrich, Helios, Singapore) and 1 mL of resin (Wizard DNA Isolation Reagent Set; Promega, San Luis Obispo, CA) were mixed with 10 mL of the processed urine (Su *et al.* 2004b). The mixture was then incubated with gentle mixing at room temperature for two hours. The resin-DNA complex was then isolated and washed with wash buffer provided by the manufacturer on minicolumns (Wizard DNA Isolation Reagent Set; Promega). Urine DNA was then eluted in 100 μL deionized free water.

3.3 Quantitative measurements of nucleic acids

3.3.1 Principle of real-time quantitative PCR

Real-time quantitative PCR systems allow robust and accurate detection of nucleic acids in human bodily fluids. In this thesis, the real-time quantitative PCR (QPCR) (Heid *et al.* 1996) was employed for quantitative measurement of DNA. Real-time quantitative reverse-transcriptase PCR (QRT-PCR) (Gibson *et al.* 1996) was employed for the quantitative measurement of mRNA and microRNA. A typical TaqMan® based real-time quantitative PCR compose of the following major components:

TaqMan® probe

A TaqMan® probe is a single-stranded synthetic oligonucleotide with a dual-labeled fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. In a reaction mixture of QPCR or QRT-PCR, if the probe is not annealed to the target sequence, it would be intact. The proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence because of energy transfer. If the target sequence is present, the probe specifically hybridises to the target sequence. The DNA polymerase, which possesses the 5' to 3' nuclease activity, cleaves the probe. The reporter and the quencher become separated, resulting in an increased fluorescence emission by the reporter. Accumulation of PCR products was detected directly by monitoring the increase in fluorescence of the reporter. The use of this type of fluorogenic probe allows great specificity as only the amplification of the intended sequence is measured, while primer-dimers and non-specific PCR products are not detected.

Sequence detector

The implementation of real-time quantitative polymerase chain reaction techniques requires suitable instrumentation capable of combining amplification, detection and quantification. All assays described in this thesis were performed on the ABI™ Prism 7300 Sequence Detector or the ABI™ Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA). These machines contain an in-built thermal cycler, a neon lamp or laser to induce fluorescence of the dye, and a charge-coupled device (CCD) camera. During real-time quantitative PCR, reactions were carried out in closed, optical tubes of a 96-well microplate. A laser/neon light was directed to each reaction via optical fibers and the resulting fluorescent emission data were collected with the CCD camera as the PCR products were being generated.

Software tool

Sequence Detection System (SDS) Software v1.2.3 (Applied Biosystems, Foster City, CA, USA) was used for the acquisition and analysis of data. Quantification of nucleic acids was achieved by detecting the corresponding threshold cycle (C_T). The C_T value is the cycle number at which the reporter signal begins to exceed a threshold above the baseline signal. This value is inversely proportional to the starting quantity of the target template.

3.3.2 One-step QRT-PCR assays for mRNA quantification

3.3.2.1 Principle

Messenger RNA was measured by one-step QRT-PCR using components provided by the TaqMan® EZ RT-PCR Kit (Applied Biosystems, Foster City, CA, USA). This is a single-tube, single enzyme quantitative system enabled with the use of the recombinant *Thermus thermophilus* (r*Tth*) DNA polymerase, which functions both as a reverse transcriptase and a DNA polymerase (Myers and Gelfand 1991). RNA is first reverse transcribed to cDNA by sequence-specific primer. Then, in the PCR amplification, the 5' to 3' nucleolytic activity of the r*Tth* DNA polymerase cleaves hybridized TaqMan® probe separating the reporter dye and quencher dye on the same probe (Holland *et al.* 1991). This results in an increase in fluorescence signal which is subsequently detected and analyzed by the sequence detector.

To allow precise quantification of mRNA in terms of copy numbers, standard curve for absolute quantification was constructed by serial dilutions of high performance liquid chromatography (HPLC)-purified single stranded synthetic DNA oligonucleotides specifying the studied amplicons. The use of synthetic DNA oligonucleotides was previously shown to be able to reliably mimic the reverse transcription step and produce standard curves that are identical to those obtained using T7-transcribed RNA (Bustin 2000).

3.3.2.2 Quantification of *human placental lactogen* (*hPL*) mRNA

In this thesis, one step real time RT-PCR was used for quantification of the *hPL* mRNA. The sequence information for the primers (Integrated DNA Technology, Coralville, IA), and probes (Proligo, Helios, Singapore) is given in Table 3.1. For the

TaqMan® probe of the *hPL* assay, the dual-labeled fluorescent probe contained 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) as the quencher dye at the 3' end. In order to avoid cross-reactivity of the assay to amplify genomic DNA, the assay was designed with a pair of intron-spanning primers.

One-step quantitative RT-PCR was set up in a reaction volume of 50 μL using the EZ rT*th* RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA). Table 3.2 summaries the preparation of the QRT-PCR reaction mixture and the thermal profile of the amplification. To each 50 μL of reaction mixture, 3 μL of extracted plasma RNA or 1 ng of extracted buffy coat/placental tissue RNA was added as template. In each analysis, calibration curve was included for absolute quantification of the target RNA (Chapter 3.3.2.1). The standard curve was constructed using serial dilutions of synthetic DNA oligonucleotides (Proligo, Singapore) on the *hPL* amplicon with concentrations ranging between 1.25×10^8 and 12.5 copies per reaction. At the same time, the analysis was run with at least four no template control reactions to monitor the level of contamination. The reaction was initiated with 2 min incubation at 50 °C for contamination control with UNG, followed by RT at 60 °C for 30 min. After deactivation of UNG at 95 °C for 5 min, the reaction was cycled for 45 times with denaturation at 94 °C for 20 s and 1 min of annealing/extension at 56 °C.

The absolute concentration of the *hPL* mRNA in each sample was calculated using one of the following formulas:

$$C_{plasma} = Q \times (V_{RNA} / V_{ext})$$

$$C_{buffy\ coat/placental\ tissue} = Q / C_{input\ RNA}$$

where C_{plasma} is the *hPL* mRNA concentration in plasma (copies/mL of plasma); Q is *hPL* mRNA quantity determined by sequence detector (copies/μL of RNA sample); V_{RNA} is the total volume of RNA solution after the RNA extraction and DNase I treatment; V_{ext} is the volume of plasma used for extraction; $C_{buffy\ coat/placental\ tissue}$ is the *hPL* mRNA concentration in buffy coat or placental tissue (copies/ng of total RNA); $C_{input\ RNA}$ is the concentration of the input total RNA determined by sequence detector (copies/ng of RNA sample).

Table 3.1 Primer, probe and standard curve sequences for QRT-PCR / QPCR assays in Chapter 3.

Target	Sequence			
hPL	F primer	5'- CATGACTCCCAGACCTCCTTC -3'	Probe	5'-(FAM) TTCTGTTGCGTTTCCTCCATGTTGG (TAMRA)-3'
(mRNA)	R primer	5'- TGCGGAGCAGCTCTAGATTG -3'		
	Standard	5'- TGCGGAGCAGCTCTAGATTGGATTTCTGTTGCGTTTCCTCCATGTTGGAGGGTGTCGGAATAGAGTCTGAGAAGCAGAAGGA GGTCTGGGAGTCATGC -3'		
SRY (63 bp)	F primer	5'-TCCTCAAAAGAAACCGTGCAT-3'	Probe	5'-(FAM)-CACCAGCAGTAACTC-(MGBNFQ)-3'
(DNA[a])	R primer	5'-AAGGACTGGATGAAAGAGGTTGTG-3'		
SRY (107 bp)	F primer	5'-TCCTCAAAAGAAACCGTGCAT-3'	Probe	5'-(FAM)-CACCAGCAGTAACTC-(MGBNFQ)-3'
(DNA[a])	R primer	5'-GGAAAGGCAAATGAATAGAGACTAAAA-3'		
SRY (377 bp)	F primer	5'-TCCTCAAAAGAAACCGTGCAT-3'	Probe	5'-(FAM)-CACCAGCAGTAACTC-(MGBNFQ)-3'
(DNA[a])	R primer	5'-TACAAAGTGGAAATATAGTTGGCTCAAGT-3'		
LEP (63 bp)	F primer	5'-CAGTCTCCTCCAAACAGAAAGTCA-3'	Probe	5'-(FAM)-CGGTTTGGACTTCA-(MGBNFQ)-3'
(DNA[a])	R primer	5'-CAGGATGGGGTGGAGCC-3'		
LEP (105 bp)	F primer	5'-CAGTCTCCTCCAAACAGAAAGTCA-3'	Probe	5'-(FAM)-CGGTTTGGACTTCA-(MGBNFQ)-3'
(DNA[a])	R primer	5'-GTCCATCTTGGATAAGGTCAGGA-3'		
LEP (356 bp)	F primer	5'-CAGTCTCCTCCAAACAGAAAGTCA-3'	Probe	5'-(FAM)-CGGTTTGGACTTCA-(MGBNFQ)-3'
(DNA[a])	R primer	5'-CTCAGCACCCAGGGCTGAG-3'		

[a] Standard curve for all DNA QPCRs were constructed from serial dilutions of genomic DNA extracted from a male human buffy coat

[b] Assay for quantification of mRNA by one-step QRT-PCR

[c] Assay for quantification of DNA by QPCR

[d] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGBNFQ, Minor-groove binding non-fluorogenic quencher

Table 3.2 One-step QRT-PCR assay for quantification *hPL* mRNA.

(A) Composition of one-step QRT-PCR reaction mixture (EZ r*Tth* RNA PCR Reagent Kit; Applied Biosystems)

Components	Volume per reaction (μL)	Final concentration
5X TaqMan EZ Buffer	10	1X
$Mn(OAc)_2$ (25 mM)	6	3 mM
dATP (10 mM)	1.5	300 μM
dCTP (10 mM)	1.5	300 μM
dGTP (10 mM)	1.5	300 μM
dUTP (20 mM)	1.5	600 μM
Forward Primer (10 μM)	1.5	300 nM
Reverse Primer (10 μM)	1.5	300 nM
Probe (5 μM)[a]	1	100 nM
AmpErase UNG (1 U/μL)[b]	0.5	0.01 U/μL
r*Tth* DNA Polymerase (2.5 U/μL)	2	0.1 U/μL
RNase-free water	18.5	-
RNA sample	3	-
Total	50	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (TAMRA, 6-carboxytetramethylrhodamine).
[b] UNG = uracil-N-glycosylase

(B) Thermal profile of one-step QRT-PCR reaction

Step		Temperature	Time
UNG treatment		50 ℃	2 min
Reverse Transcription		60 ℃	30 min
Deactivation of UNG		95 ℃	5 min
45 Cycles	Denaturation	94 ℃	20 s
	Annealing / Extension	56 ℃	1 min

3.3.3 Two-step QRT-PCR assays for microRNA quantification

3.3.3.1 Principle

MicroRNA in RNA samples was measured by a two-step QRT-PCR assay using components provided by the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). This two-step QRT-PCR system involves two separate procedures (Figure 3.2).

Reverse Transcription (RT)

In this step, a stem-loop primer hyrbridizes to the 3' end of the mature miRNA. It can distinguish between other highly similar mature miRNA sequences (Figure 3.2). The primer can also distinguish between mature miRNAs with single base difference (Applied Biosystems; Product bulletin on TaqMan MicroRNA Assays: Quantitate microRNAs with the specificity and sensitivity of TaqMan assay chemistry; accessed 3 October 2006). Upon RT, cDNA of the microRNA is generated as an extension from the 3' end of the stem-loop primer, resulting in a cDNA-DNA hybrid for subsequent PCR amplification.

The Multiscribe™ reverse transcriptase used in the RT step is capable of generating cDNA from both microRNA and single-strand DNA. Hence, standard curve for absolute quantification was constructed by serial dilutions of single-stranded synthetic DNA oligonucleotides specifying the studied miRNAs. According to the results described in Chapter 4.3.1, the use of synthetic DNA oligonucleotides is able to reliably mimic the RT step and produce standard curves that are identical to synthesized RNA oligonucleotides. Since synthetic DNA oligonucleotides are less expensive and the commercial turn around time for their synthesis is relatively short,

they were used as the standard curves for my experiments.

PCR Amplification

The quantity of cDNA-DNA hybrid generated is directly proportional to the initial quantity of miRNA present in the RNA sample. Hence, the assay quantifies the miRNA using primers and probe amplifying the cDNA-DNA hybrid from the RT step (Figure 3.2). The forward primer hybridizes specifically to the cDNA portion of the microRNA in the hybrid; the reverse primer hybridizes specifically to the unfolded stem-loop sequence of the stem-loop primer in the hybrid; the probe falls on the junction within the hybrid. Such design further enhances the specificity of the assay to miRNA detection. It is also capable of generating a dynamic range of detection of up to seven logs.

3.3.3.2 Advantages

The two-step QRT-PCR system for microRNA quantification involves the use of a stem-loop primer and a set of specific primers and probes which has several important features for the detection of microRNA.

Detection of the very short microRNA

QRT-PCR or QPCR requires a template of at least 60 nucleotides for primers and probe hybridization and efficient PCR amplification. However, mature miRNAs are very short, of about 21-25 bases long. They, alone, cannot provide enough target sequence length for primers and probe hybridization. The use of a hairpin primer for the generation of a cDNA-DNA hybrid provides a target template long enough for the subsequent PCR amplification, which is otherwise impossible.

Increased specificity

The use of hairpin primer for the QRT-PCR has increased the specificity of the assay in several aspects. First, the hairpin primer recognizes and hybridizes to RNA with the exact 3' end sequence. Even a single base extension at the 3' end will result in a delayed threshold cycle in the detection. This allows the primer to distinguish the mature miRNA target from its miRNA precursors or any genomic DNA contaminants in the RNA samples. Second, the primers and probes used in the PCR amplifications are very specific to the cDNA-DNA hybrid. This has greatly enhanced the specificity of the assay to the target microRNA and allows the assay to differentiate between highly similar microRNA sequences with even a single nucleotide difference.

3.3.3.3 TaqMan® MicroRNA Assays

TaqMan® MicroRNA Assays (Applied Biosystems) were used for quantification of mature microRNA sequences. The assays were designed by and purchased from Applied Biosystems for different microRNA targets. The assay IDs and sequences of the target microRNAs used in this thesis are summarized in APPENDIX I. For the TaqMan® probes of all the assays, the dual-labeled fluorescent probe contained 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and minor-groove binding (MGB) non-fluorogenic quencher (NFQ) at the 3' end.

The first step, reverse transcription (RT), was set in a reaction volume of 7.5 μL using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Table 3.3 A and C summarize the preparation of the RT reaction mixture and the thermal profile, respectively. To each 7.5 μL of the reaction mixture, 2.5 μL of extracted plasma RNA or 2.5 ng of extracted buffy coat/placental tissue RNA was added as template. In each analysis, a calibration curve was included for absolute

quantification of the target RNA. The standard curve was constructed using serial dilution of synthetic DNA oligonucleotides (Proligo) on the target microRNA with concentrations ranging between 6.25 x 10^6 and 62.5 copies per RT reaction. At the same time, the analysis was run with at least four no template control reactions to monitor the level of contamination in reagents used in the RT reaction. The reaction was initiated with a 5-min incubation at 4 °C for initial hybridization between primers and target sequences. After hybridization at 16 °C for 30 min, RT was initiated at 42 °C for another 30 min. Deactivation of the reverse transcriptase was performed at 85 °C for 5 min.

The second step, PCR amplification, was set up in a reaction volume of 20 μL using TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA). Table 3.3 B and D summarize the preparation of the PCR amplification reaction mixture and the thermal profile, respectively. To each 20 μL of reaction mixture, 1.33 μL of RT product from all the reactions was added as template. At the same time, the analysis was run with another four no template control reactions to monitor the level of contamination in the reagents used in the PCR reaction.

The reaction was initiated with 10 min incubation at 95 °C for AmpliTaq Gold® enzyme activation and initial DNA denaturation. Then, the reaction was cycled for 40 times with denaturation at 95 °C for 15 sec and 1 min of annealing/extension at 60 °C.

The absolute concentration of the target sequences in each sample was calculated using one of the following formulas:

$$C_{plasma} = Q \times (V_{RNA} / V_{ext})$$

$C_{buffy\ coat/placental\ tissue} = Q / C_{input\ RNA}$

where C_{plasma} is the target sequence concentration in plasma (copies/mL of plasma); Q is target sequence quantity determined by sequence detector (copies/μL of RNA sample); V_{RNA} is the total volume of RNA solution after the RNA extraction and DNase I treatment; V_{ext} is the volume of plasma used for extraction; $C_{buffy\ coat/placental\ tissue}$ is the target sequence concentration in buffy coat or placental tissue (copies/ng of total RNA); $C_{input\ RNA}$ is the concentration of the input total RNA determined by the sequence detector (copies/ng of RNA sample).

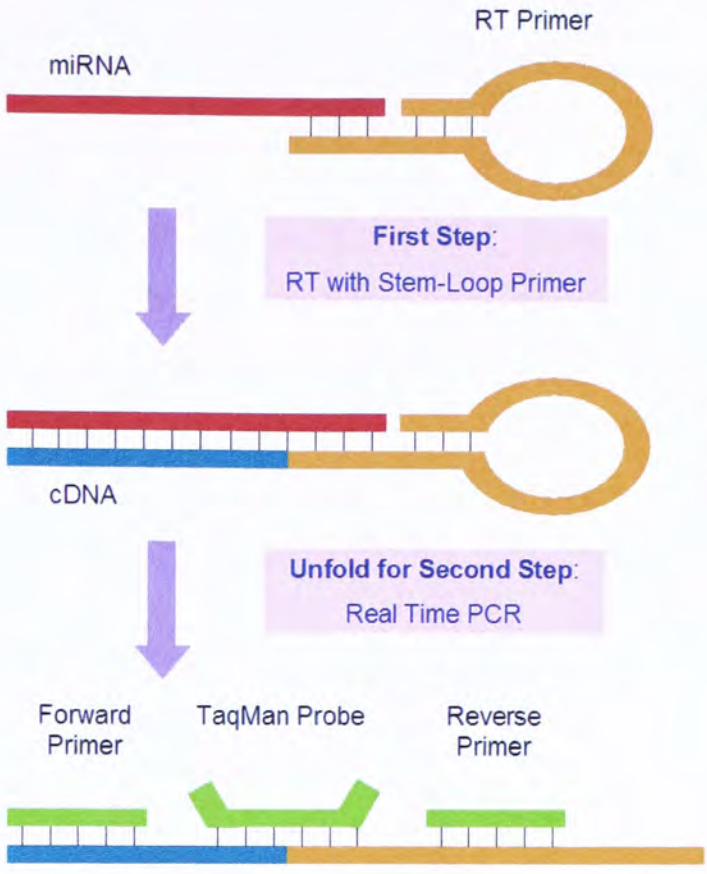


Figure 3.2 Two-step QRT-PCR assays for miRNA quantification.

In the RT step, a stem-loop primer hyrbridizes specifically to the 3' end of the mature miRNA. Upon reverse transcription, cDNA of the miRNA is generated as an extension from the 3' end of the stem-loop primer, resulting in a cDNA-DNA hybrid for subsequent PCR amplification. The forward primer hybridizes specifically to the cDNA of the miRNA in the hybrid; the reverse primer hybridizes specifically to the unfolded stem-loop sequence of the stem-loop primer in the hybrid; the probe falls on the junction within the hybrid. QPCR was then performed for real-time detection. (Modified from TaqMan® MicroRNA Assays instruction manual, Applied Biosystems, 2005)

Table 3.3 Two-step QRT-PCR assay for microRNA quantification.

(A) Composition of reverse transcription (RT) reaction mixture

Component	Volume for one reaction (μL)	Final concentration
10X RT Buffer[a]	0.75	1X
dNTPs (with dTTP; 100 mM)[a]	0.08	13.33 mM
5X Stem-loop RT Primer[b]	1.5	1X
RNase Inhibitor (20 U/μL)[a]	0.09	2.67 U/μL
MultiScribe™ Reverse Transcriptase (50 U/μL)[a]	0.5	6.67 U/μL
Nuclease-free water	2.08	-
RNA sample	2.5	-
Total	7.5	-

[a] Components provided by TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems
[b] Components provided by TaqMan® MicroRNA Assay, Applied Biosystems

(B) Composition of PCR reaction mixture

Component	Volume for one reaction (μL)	Final concentration
TaqMan 2X Universal PCR Master Mix (No AmpErase UNG)[b,c]	10	1X
TaqMan MicroRNA Assay Mix (10X)[d]	1	1X
Nuclease-free water	7.67	-
RT Product	1.33	-
Total	20	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (MGB NFQ, minor-groove binding non-fluorogenic quencher).
[b] UNG = uracil-N-glycosylase
[c] Applied Biosystems
[d] Components provided by TaqMan® MicroRNA Assay, Applied Biosystems

(C) Thermal profile of reverse transcription (RT)

Step	**Temperature**	**Time**
Initial incubation	4 ℃	5 min
Annealing of stem-loop RT primers	16 ℃	30 min
Reverse transcription	42 ℃	30 min
Deactivation of MultiScribe™ Reverse Transcriptase	85 ℃	5 min
Final incubation	4 ℃	∞

(D) Thermal profile of PCR amplification

Step		**Temperature**	**Time**
Activation of AmpliTaq Gold®		95 ℃	10 min
40 Cycles	Denaturation	95 ℃	15 s
	Annealing / Extension	60 ℃	1 min

3.3.4 QPCR assays for DNA quantification

3.3.4.1 Principle

DNA in samples was measured by QPCR using components provided by the TaqMan® PCR Core Reagent Kit (Applied Biosystems, Foster City, CA). The principle behind the quantification is similar to that of QRT-PCR, except, in QPCR, the AmpliTaq Gold® DNA polymerase is used and there is no reverse transcription step is involved. Moreover, standard curve for absolute quantification was constructed by serial dilutions of human male genomic DNA extracted from blood cells.

3.3.4.2 Quantification of the *leptin* gene and the *sex-determining region on Y chromosome* gene

QPCR assays were used for the quantification of the *leptin* (*LEP*) gene and the *sex-determining region on Y chromosome* (*SRY*) gene in different bodily fluids. For each of the *LEP* and *SRY* genes, three assays of different amplicon sizes were used. The sequence information for the primers (Integrated DNA Technology) and probes (Applied Biosystems) is given in Table 3.1. For the TaqMan® probes of all assays, the dual-labeled fluorescent probe contained 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and the minor-groove binding (MGB) non-fluorogenic quencher (NFQ) at the 3' end.

QPCR was set up in a reaction volume of 50 μL using the TaqMan® PCR Core Reagent Kit (Applied Biosystems). Table 3.4 summaries the preparation of the QPCR reaction mix and the thermal profile of the amplification. To each 50 μL of reaction mixture, 5 μL of extracted urine/plasma DNA was added as a template. In

each analysis, a calibration curve was included for absolute quantification of the target DNA (Chapter 3.3.2.1). The standard curve was constructed using serial dilutions of male blood cells genomic DNA with concentrations ranging between 0.78 and 10,000 genome equivalents per five microliters. At the same time, the analysis was run with at least four no template control reactions to monitor the level of contamination. The reaction was initiated with 2 min incubation at 50 °C for contamination control with UNG. After deactivation of UNG at 95 °C for 10 min, the reaction was cycled for 50 times with denaturation at 95 °C for 30 s and 1 min of annealing/extension at 60 °C.

The absolute concentration of the DNA in each sample was calculated using the following formula:

$$C_{plasma/urine} = Q \times (V_{DNA} / V_{ext})$$

where $C_{plasma/urine}$ is the DNA concentration in plasma/urine (copies/mL of plasma/urine); Q is DNA quantity determined by the sequence detector (copies/μL of DNA sample); V_{DNA} is the total volume of DNA solution after the DNA extraction; V_{ext} is the volume of plasma used for extraction.

Table 3.4 QPCR assay for DNA quantification of *LEP* and *SRY* DNA.

(A) Composition of QPCR reaction mixture (TaqMan® PCR Core Reagent Kit, Applied Biosystems)

Component	Volume for one reaction (μL)	Final concentration
10X TaqMan Buffer A	5	1X
$MgCl_2$ (25 mM)	8	4 mM
dATP (10 mM)	1	200 μM
dCTP (10 mM)	1	200 μM
dGTP (10 mM)	1	200 μM
dUTP (20 mM)	1	400 μM
Forward Primer (10 μM)	5	1 μM
Forward Primer (10 μM)	5	1 μM
Probe (10 μM)[a]	2.5	500 nM
AmpliTaq Gold® (5 U/μL)	2	0.025 U/ μL
AmpErase UNG (1 U/μL)[b]	0.5	0.01 U/ μL
Dimethyl sulfoxide (DMSO)	2.5[c]	50 nL/ μL
Deionized water	10.5	-
DNA sample	5	-
Total	50	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (MGB NFQ, minor-groove binding non-fluorogenic quencher).
[b] UNG = uracil-N-glycosylase
[c] DMSO is added in all *LEP* assays; DMSO is replaced with deionized water in all *SRY* assays

(B) Thermal profile

Step		**Temperature**	**Time**
UNG treatment		50 ℃	2 min
Deactivation of UNG and Activation of AmpliTaq Gold®		95 ℃	10 min
50 Cycles	Denaturation	95 ℃	30 s
	Annealing / Extension	58 ℃	1 min
Final incubation		72 ℃	1 min

3.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

3.4.1 Principle

The MassARRAY® system (Sequenom, San Diego, CA) is an accurate and high-throughput technology for DNA/RNA genotyping. It is capable of resolving single nucleotide polymorphisms (SNPs) of DNA/RNA samples quantitatively, by utilizing the MALDI-TOF MS to differentiate mass difference of products generated from the homogeneous MassEXTEND™ (hME) assay (Sequenom, San Diego, CA).

The hME assay consists of an initial PCR and a primer extension reaction. In the initial PCR, a single pair of primers flanking the region with the SNPs is used. Upon amplification, the PCR products of different genotypes of the SNP are produced in proportion to the genotypic ratio in the original nucleic acid sample. In the primer extension reaction, an oligonucleotide primer (extension primer) anneals to a position right beside the SNP of interest. Thermal cycling was then performed using a reaction mixture with a DNA polymerase along with a mixture of terminator nucleotides, a combination of different deoxyribonucleotides (dNTP) and dideoxynucleotides (ddNTP), which allows extension of the hME primer through the polymorphic site and generates allele-specific extension products with different unique molecular masses. MALDI-TOF MS is then used to analyze the resultant masses of the extension products and to report genotype of the nucleic acid samples (Tang *et al.* 1995).

3.4.2 *Zinc finger protein gene* assay for determining the fractional concentration of male DNA

Zinc finger protein gene assay was used to determine the fractional concentration of male DNA in a mixture of male and female DNA. The fractional concentration was expressed as a percentage of male DNA in a mixture. In principle, the assay uses the MassARRAY® system (Sequenom) to determine the genotypic ratio of a single base difference, which is stably inherited outside the pseudoautosomal region in the X- and Y-chromosome, in a mixture of male and female DNA. The base is inherited as the nucleotide T on the *X-linked zinc finger protein gene* (*ZFX*); it is inherited as the nucleotide C on the *Y-linked zinc finger protein gene* (*ZFY*). Making use of such allelic ratio, the proportion of male to female DNA in the DNA mixture can be determined. The PCR primers and the extension primer were generated by the MassARRAY assay design software, SpectroDESIGNER (Sequenom). The PCR and extension primers employed and the thermal profile of the assay are summarized in **Table 3.5**.

The initial PCR was set up in a reaction volume of 25 μL using the PCR components provided with AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA). Table 3.6 A and D summarize the preparation of the PCR reaction mixture and the thermal profile of the amplification, respectively. A 10-mer tag with the sequence of 5'-ACGTTGGATG-3' was added to the 5' end of the forward and reverse PCR primers so that the masses of the primers would fall out of the detection range of the mass spectrometer. To each 25 μL of reaction mixture, 5 μL of extracted urine/plasma DNA or 50 ng of extracted buffy coat/urine cell-pellet DNA was added as template. At the same time, the analysis was run with at least four no template

control reactions to monitor the level of contamination. The reaction was initiated at 95°C for 15 min, followed by denaturation at 95°C for 20 s, annealing at 56°C for 30 s, extension at 72°C for 1 min for 45 cycles, and a final incubation at 72°C for 3 min.

Shrimp alkaline phosphatase (SAP) treatment was then set up to remove unincorporated dNTPs in the PCR product. Table 3.6 B and D summarize the preparation of the SAP reaction mixture and the thermal profile, respectively. The reaction was initiated incubated at 37°C for 40 min to activate SAP activities, followed by 85°C for 5 min for SAP deactivation.

The hME reaction was then set up for primer extension. Table 3.6 C and F summarize the preparation of the hME reaction mixture and the thermal profile, respectively. The reaction was initiated at 94°C for 2 min, followed by 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s for 75 cycles. Then, 24 μL of water and 12 mg of the SpectroCLEAN Resin (Sequenom) were added to the extension products for a final clean up. The mixtures were mixed on a rotator for 20 min, followed by a centrifugation step at 361 *g* for 5 min. 15 to 25 nL of the final products were dispensed onto a SpectroCHIP (Sequenom) by a MassARRAY Nanodispenser S (Sequenom). Data acquisition from the SpectroCHIP was done in the MassARRAY Analyzer Compact Mass Spectrometer (Sequenom). Mass data were imported into the MassARRAY Typer (Sequenom) software for analysis.

For the *ZFX* with an allelotype T, the primer extension product would have a sequence read as 5'- TCATCTGGGACTGTGCAA -3' with a molecular mass of 5498.6 Dalton (Da) For the *ZFY* with an allelotype C, the sequence of the primer extension product would be 5'- TCATCTGGGACTGTGCAGT -3' with a molecular mass of 5818.8 Da. This mass difference would be illustrated as two distinct peaks

which were resolvable by the mass spectrometer. The peak ratio of the two allelotypes could be used to estimate the ratio of the copy of *ZFX* to *ZFY*, which in turn could be used to estimate the ratio of X- to Y-chromosome. Because each male has one of both the X- and Y-chromosome while each female has two X-chromosomes, the percentage of male DNA in a mixture of male and female DNA was calculated using the following formulas:

$$\text{Male DNA \%} = (2 \times H_Y) / (H_X + H_Y) \times 100\%$$

where H_X is the peak height of the *ZFX* reported by MALDI-TOF MS and H_Y is the peak height of the *ZFY* reported by MALDI-TOF MS.

Table 3.5 Primer and product sequences for the *zinc finger protein gene* assay.

Primer	*Sequences (5' to 3')*	*Mass (Da)*
Forward PCR primer	**ACGTTGGATG**GCTAAAACATCATCTGGGAC[a]	NR[b]
Reverse PCR primer	**ACGTTGGATG**TCATTCCTGAGCAAGTGCTG[a]	NR[b]
Extension primer	TCATCTGGGACTGTGCA	5201.4
Extension product 1	TCATCTGGGACTGTGCAA	5498.6
Extension product 2	TCATCTGGGACTGTGCAGT	5818.8

[a]Bold font indicates the 10-mer tags added to the 5' end of the primers in such a way that the masses of the primers would fall out of the analytical range of the mass spectrometer.
[b]NR = not relevant.

Table 3.6 *Zinc finger protein gene* assay for quantification of male DNA percentage in a mixture of male and female DNA.

(A) Composition of initial PCR reaction mixture (AmpliTaq Gold® DNA Polymerase, Applied Biosystems)

Component	Volume for one reaction (μL)	Final concentration
10X PCR buffer II	2.5	1X
$MgCl_2$ (25 mM)	4	4 mM
dATP (10 mM)	0.625	250 μM
dCTP (10 mM)	0.625	250 μM
dGTP (10 mM)	0.625	250 μM
dUTP (20 mM)	0.625	500 μM
Forward Primer (10 μM)	0.5	200 nM
Forward Primer (10 μM)	0.5	200 nM
AmpliTaq Gold® DNA Polymerase(5 U/μL)	0.25	0.05 U/ μL
Deionized water	9.75	-
DNA sample	5	-
Total	25	-

(B) Composition of shrimp alkaline phosphatase (SAP) treatment mixture (Sequenom)

Component	Volume for one reaction (μL)
MassARRAY™ hME buffer	0.34
SAP	0.6
Deionized water	3.04
PCR product	25
Total	29

(C) Composition of homogenous MassEXTEND™ reaction mixture *(Sequenom)*

Component	**Volume for one reaction (μL)**	***Final concentration***
ddATP (8.96 mM)	*0.1*	*64 μM*
ddTTP (8.96 mM)	*0.1*	*64 μM*
ddCTP (8.96 mM)	*0.1*	*64 μM*
dGTP (8.96 mM)	*0.1*	*64 μM*
Extension Primer (10 μM)	*2.16*	*1542.86 nM*
Thermosequenase (32.94 U/μL)	*0.036*	*0.08 U/μL*
Deionized water	*1.404*	-
SAP-treated PCR product	*10*	-
Total	*14*	-

(D) Thermal profile of initial PCR

Step		**Temperature**	**Time**
Activation of AmpliTaq Gold®		*95 ℃*	*10 min*
	Denaturation	*95 ℃*	*30 s*
45 Cycles	Annealing	*55 ℃*	*30 s*
	Extension	*72 ℃*	*30 s*
Final incubation		*72 ℃*	*30 s*

(E) Thermal profile of shrimp alkaline phosphatase (SAP) treatment

Step	**Temperature**	**Time**
Active SAP activity	37 ℃	40 min
Deactivation of SAP	85 ℃	5 min

(F) Thermal profile of homogenous MassEXTEND™ reaction

Step		**Temperature**	**Time**
Activation of AmpliTaq Gold®		94 ℃	2 min
	Denaturation	94 ℃	5 s
75 Cycles	Annealing	52 ℃	5 s
	Extension	72 ℃	5 s

3.5 Statistical analyses

Statistical analyses were performed with SigmaStat 3.0 software (SPSS).

SECTION III: CIRCULATING PLACENTAL MICRORNAS IN MATERNAL PLASMA AS MARKERS FOR PRENATAL DIAGNOSIS

CHAPTER 4: THE EXISTENCE AND QUANTITATIVE DETECTION OF CELL-FREE MICRORNAS IN PLASMA

4.1 Introduction

The discovery of fetal nucleic acids in the plasma of pregnant women (Lo *et al.* 1997, Ng *et al.* 2003b, Lo and Chiu 2007) has led to the development of a number of noninvasive prenatal diagnostic tests. Circulating fetal DNA in maternal plasma has been used for prenatal investigations of fetal rhesus D status (Faas *et al.* 1998, Lo *et al.* 1998a), sex-linked diseases (Chiu *et al.* 2002b, Costa *et al.* 2002a) and β-thalassemia (Chiu *et al.* 2002b). However, these applications rely on the Y-chromosome sequences or the paternally-inherited polymorphisms or mutations for the detection of fetal DNA in maternal plasma, and thus would not be applicable to all pregnancies. Using the epigenetic difference between DNA in the placenta and the maternal blood cells, our group has developed a universal fetal DNA marker (Chim *et al.* 2005), which is gender- and polymorphism-independent. Furthermore, our group has also discovered that placental mRNA transcripts are released into maternal plasma and developed them as another class of gender- and polymorphism-independent fetal markers. Quantitative aberrations of these fetal-derived placental mRNA transcripts in maternal plasma have been shown in conditions such as preeclampsia (Ng *et al.* 2003a) and fetal aneuploidies (Ng *et al.* 2004). The feasibility of detecting fetal chromosomal aneuplodies noninvasively, using a fetal-derived *PLAC4* mRNA transcript in maternal plasma, has been demonstrated (Lo *et al.* 2007). These findings have heralded a new generation of molecular markers for noninvasive prenatal diagnosis.

Recent studies on microRNAs (miRNAs) offer possibilities for developing a new class of molecular markers. MiRNAs are short (21-25 nucleotides), single-stranded and non-protein coding RNAs (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee and Ambros 2001). They possess important roles in regulating gene expression through binding to the 3' untranslated region of the target mRNAs (Lai 2002). These regulations are involved in functions such as development (Krichevsky *et al.* 2003), differentiation (Esau *et al.* 2004), apoptosis (Baehrecke 2003) and oncogenesis (Michael *et al.* 2003, Calin *et al.* 2005). Although ribonuclease activity has been observed in plasma (Reddi and Holland 1976), previous data have shown that plasma mRNAs are surprisingly stable (Ng *et al.* 2002), possibly through association with subcellular particles. Here, I hypothesised that microRNAs should also exist in plasma because, like mRNA, miRNAs are also present at high copy numbers (Lim *et al.* 2003) and exist naturally in the cytoplasm (Yi *et al.* 2003, Lund *et al.* 2004). Thus, they may be released into plasma readily upon apoptosis. However, no data have been reported on whether miRNAs in human plasma might be stable enough for detection at the time of this writing.

This part of the thesis aims to address the unresolved area outlined above. Here, I hypothesized that cell-free miRNAs exist stably in human plasma and can be robustly extracted for the subsequent quantification. I tested this hypothesis by first evaluating the use of a newly devised plasma RNA extraction protocol (Chapter 3.2.1) and the use of the TaqMan® MicroRNA Assay as a two-step real time QRT-PCR (Chapter 3.3.3) for the absolute quantification of the mature miR-16. MiR-16 is a miRNA highly expressed in many human tissues (Barad *et al.* 2004). In particular, numerous reports have shown its presence in lymphocytes (Calin *et al.* 2002, Calin *et al.* 2005). Therefore, it is highly probably to be present in plasma. The detection of

miRNA in plasma would open up new opportunities in the development of a novel class of circulating nucleic acid for noninvasive diagnosis of different diseases and conditions.

4.2 Materials and methods

4.2.1 Sample collection

Peripheral blood samples were collected from pregnant women, with singleton uncomplicated pregnancies, just before elective cesarean delivery. Paired term placental tissues were also collected just after delivery. They were all in the third trimester of pregnancy. In addition, peripheral blood samples were collected from healthy volunteers with informed consent. All the samples were prepared using the protocol described in Chapter 3.1. All pregnant women were recruited with informed consent for the Department of Obstetrics and Gynaecology, Prince of Wales Hospital, Shatin, Hong Kong. The study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee.

4.2.2 Experimental design

Validations of the two-step QRT-PCR system for miRNA quantification

Experiment 1

To evaluate the quantitative linearity of the assay and to address the sensitivity of the assay, I constructed a standard curve using a serial dilution of synthetic RNA oligonucleotides (Proligo) of miR-16. In a standard curve, the regression correlation coefficient (R^2) demonstrates the degree of correlation between the RNA concentration and the threshold cycle (C_T) generated from amplification of the target

sequence. It is expected that, if the two-step QRT-PCR system is to be used for absolute quantification, it should have a high R^2 within the range of detection. To investigate these parameters of the assay, a standard curve was constructed using a serial dilution of synthetic RNA oligonucleotides (Proligo) of the target sequences from 6.25 x 10^9 to 62.5 copes per reverse transcription (RT) reaction as described in Chapter 3.3.3.3. The R^2 and detection limits were used as the reference of analysis.

Experiment 2

To evaluate the precision of the miRNA quantification assay, I replicated the detection of 500 pg per RT reaction of a placental RNA preparation extracted and certified to contain miRNAs by a company (Ambion®, Austin, TX) for 20 times as described in Chapter 3.3.3.3. C_T values and intra-assay coefficient of variation (CV%) of C_T values were used as the reference of analysis. It was expected that if the assay was highly reproducible, the assay would give very similar C_T values and a very low analytical intra-assay CV%.

Experiment 3

Experiment 3 was performed to give reference for the choice of oligonucleotides as calibrator. The TaqMan® MicroRNA Assay (Applied Biosystems) involves the use of MultiScribe™ reverse transcriptase which is a recombinant Moloney Murine Leukemia virus (rMoMuLV) reverse transcriptase that can reverse transcribe both DNA and RNA oligonucleotides. This experiment was performed to investigate if both DNA and RNA oligonucleotides were suitable to be used as calibrator to produce similar PCR efficiencies. The use of DNA, instead of RNA, oligonucleotides as calibrator would greatly reduce the cost of the experiments, especially in the

subsequent larger-scale experiments as described in Chapter 5. To evaluate the PCR efficiency of the assay on DNA and RNA oligonucleotides, I constructed a standard curve using a serial dilutions of synthetic DNA and RNA oligonucleotides (Proligo) of miR-16. In a standard curve, the gradient demonstrates the efficiency of the RT-PCR of the target sequence. It was expected that, if both DNA and RNA oligonucleotides could serve as a calibrator, they would produce similar PCR efficiencies. To investigate this parameter of the assay, a standard curve was constructed using serial dilutions of both synthetic DNA and RNA oligonucleotides (Proligo) of the target sequences from 6.25 x 10^9 to 62.5 copes per RT reaction as described in Chapter 3.3.3.3. The gradients and C_T of corresponding calibrators were used as the reference of analysis.

Experiment 4

To evaluate the specificity of the assay to the exact mature miRNA sequence, rather than the miRNA precursor sequences, I compared the detection of three DNA oligonucleotides coding the exact sequences of miR-16, miR-16 with one base extension and miR-16 with fifteen bases extension at the 3' end (Table 4.1). It was expected that if the assay was specific to the mature miR-16 sequence, no or delayed amplification signal of the assay would be detected for the 2 oligonucleotides with 3' extensions, compared to the one coding for only the exact miR-16 sequence. To do so, these DNA oligonucleotides (Table 4.1), diluted to 2.5 x 10^5 copies/μL, were measured with the assay as described in Chapter 3.3.3.3. The C_T of the detection was used as the reference of analysis.

Experiment 5

This experiment was performed to evaluate the specificity of the assay for mature

miRNA sequence, rather than any fragmented genomic DNA, carried over as contaminants of RNA extraction, with similar sequence to the short miRNA. Total RNA extracted from maternal blood cells as described in Chapter 3.2.1 was divided into four equal aliquots. They were subjected to different combinations of DNase I digestion (Invitrogen, Carlsbad, CA) and RNase A digestion (Fermentas, Hanover, MD) as shown in Figure 4.1. They were then quantified for miR-141 using the two-step QRT-PCR system as described in Chapter 3.3.3.3.

Feasibility on the detection of miRNA in plasma

Feasibility test was performed to verify the possibility of detecting of cell-free miRNAs in maternal plasma. Plasma samples from 20 healthy pregnant women in their third trimester of pregnancy were tested for the presence of cell-free miR-16 in the processed plasma using the two-step QRT-PCR system as described in Chapter 3.3.3.3. In addition, the precision of this analytical system involving both miRNA extraction and miRNA quantification was addressed. A plasma sample was obtained from a randomly chosen healthy non-pregnant woman, divided into 18 identical aliquots, each of 0.8 mL, for 18 replicated miRNA extractions, and quantified for the concentration of miR-16. The CV% for the C_T of the two-step QRT-PCR was calculated.

4.2.3 RNA extraction and quantification

Processing of peripheral blood samples for cell-free plasma followed the procedure as described in Chapter 3.1.1. Two-step QRT-PCR assays for miR-16 quantification followed the procedure as described in Chapter 3.3.3.3. TaqMan® MicroRNA Assay of miR-16 was ordered from Applied Biosystems. Serial dilutions of synthetic oligonucleotides were used for the absolute quantification of the miR-16.

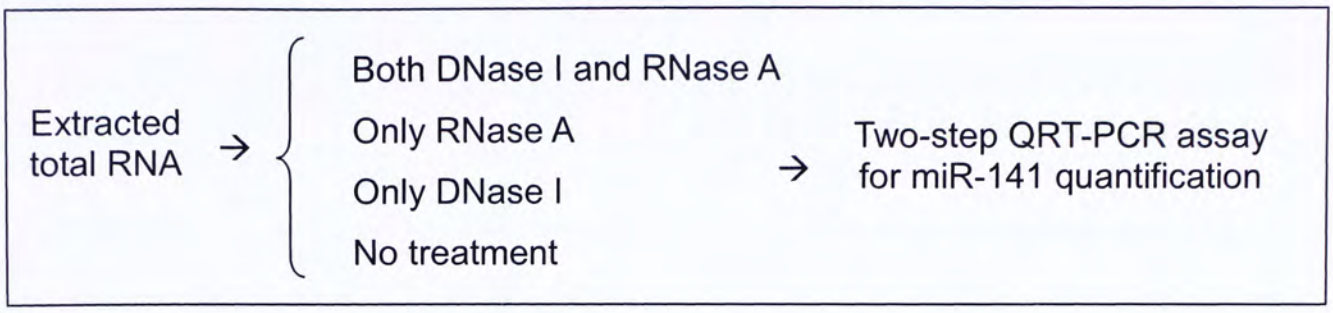


Figure 4.1 Experimental procedures for evaluating the specificity of the two-step QRT-PCR assay in detecting RNA, but not DNA.

Extracted RNA was divided into four equal aliquots which were subsequently subjected to different combinations of DNase I and/or RNase A treatments. The concentrations of miR-141 after different treatments or no treatment were quantified by the two-step QRT-PCR assay.

Table 4.1 DNA oligonucleotide sequences coding for miR-16 with and without 3' base extensions used for studying the specificity of the two-step QRT-PCR assay.

Primer	Sequences (5' to 3')
miR-16	TAGCAGCACGTAAATATTGGCG
miR-16 + 1 base	TAGCAGCACGTAAATATTGGCG**A**
miR-16 + 15 bases	TAGCAGCACGTAAATATTGGCG**CAGCTTAACGATATT**

[a]Bold font indicates the base extensions at the 3' end

4.3 Results

4.3.1 Validation of two-step QRT-PCR system for miRNA quantification

To assess the linearity and sensitivity of the assay, serial dilutions of the synthetic miR-16 RNA oligonucleotide were analyzed using the two-step QRT-PCR assay. The standard curve demonstrated that this quantification system had a dynamic range between 6.25 x 10^9 and 62.5 copies of miR-16 per RT reaction and had an R^2 of 0.99 (Figure 4.2). This assay could detect down to 62.5 copies of target sequence per RT reaction.

To assess the precision of the two-step QRT-PCR assay, 20 replicated quantifications of miR-16 in 500 pg per RT reaction of an miRNA-containing placental RNA preparation (Ambion®, Austin, TX) were performed. The CV% for the C_T values of these replicated QRT-PCR was 0.53% (Mean±SD, 29.52±0.16).

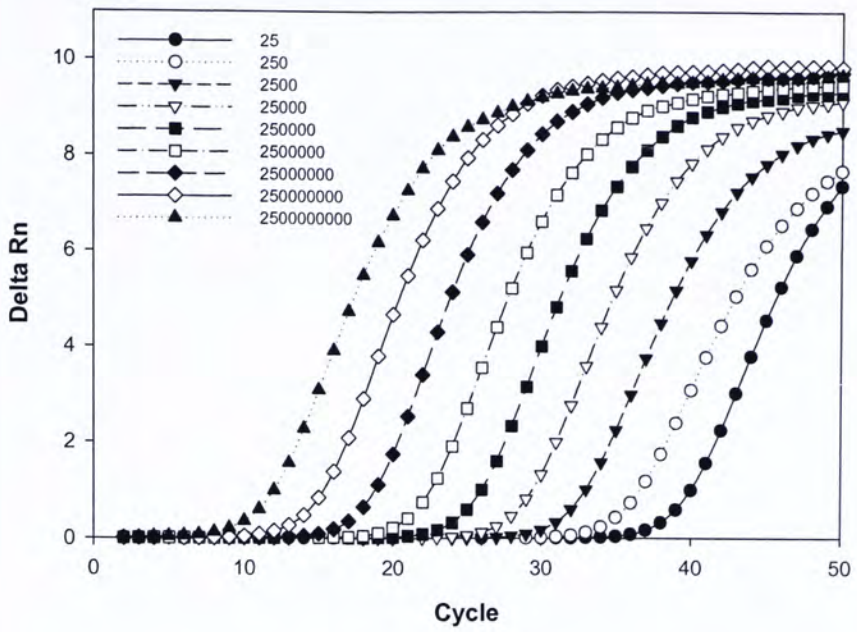
To investigate if both DNA and RNA oligonucleotides would be suitable to be used as calibrators, PCR efficiencies were compared between the detections of serially diluted synthetic DNA and RNA oligonucleotides (Proligo) of miR-16. The gradients were -3.55 and -3.56 for the standard curves of DNA and RNA, respectively (Figure 4.3). The R^2 for both of them was 0.99. The C_T of corresponding calibrators was similar between the standard curves of DNA and RNA. This assay could detect down to 62.5 copies of target sequence per RT reaction.

To evaluate the specificity of the assay for the mature miRNA sequence, the C_T values as given by the two-step QRT-PCR assay of DNA oligonucleotides coding for the exact miR-16 sequence without any base extension, miR16 + 1 base, and miR-16

+ 15 bases at the 3' end, at the same concentrations were compared. At the same detection threshold, the C_T values were 22.2, 24.0, and 36.1 for the oligonucleotides coding for miR-16, miR-16 + 1 base, and miR-16 + 15 bases, respectively. The C_T values of the detection of miR-16 + 1 base and miR-16 + 15 bases at 3' end were about 1.8 and 13.9 C_T, respectively, delayed, compared with that of miR-16 without any base extension (Figure 4.4).

To evaluate the specificity of the assay for the the mature miRNA sequence, rather than any fragmented genomic DNA, I subjected 4 aliquots of an RNA preparation to no treatment or treatment by DNase I and/or RNase A. With no treatment, miR-141 was readily detected in this RNA preparation. However, with RNase A treatment, it was shown that no or extremely low quantities of miR-141 could be detected. The presence of DNase I treatment did not largely affect the quantities of miR-141, compared to the corresponding treatments without DNase I. The data suggested that the two-step QRT-PCR assays were truly RNA-specific (Table 4.2).

(A)



(B)

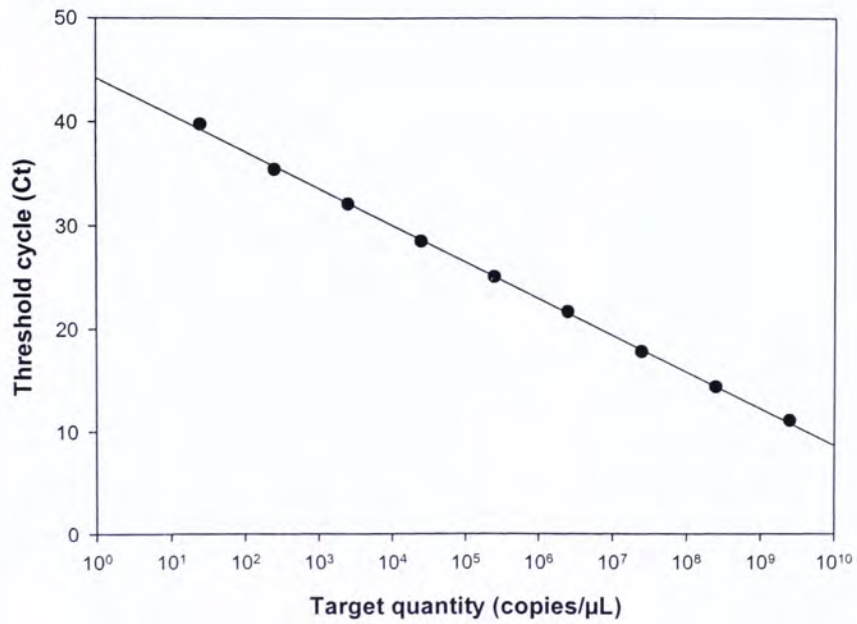
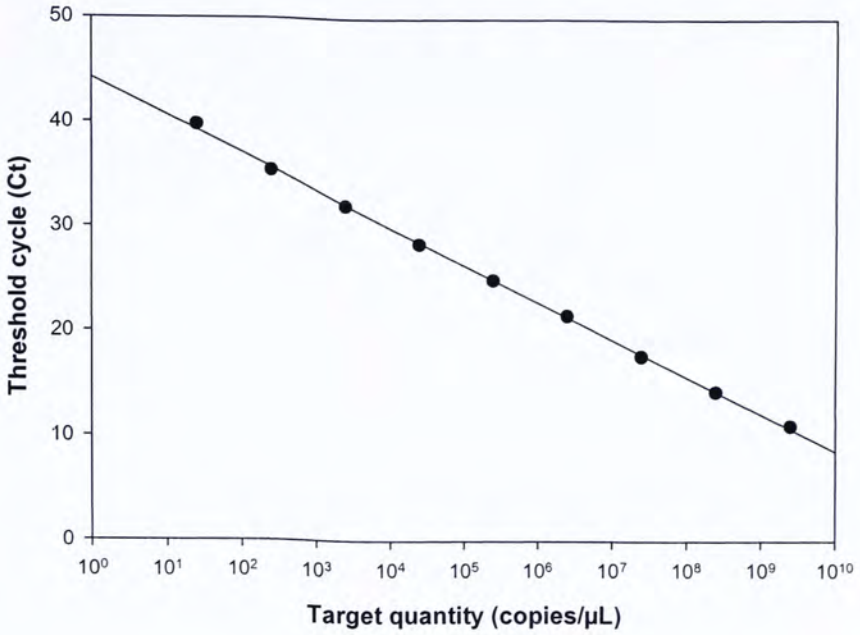


Figure 4.2 Two-step QRT-PCR for miR-16.

(A), amplification plots obtained using two-step QRT-PCR for the miR-16 RNA oligonucleotide. Each plot corresponds to a particular input target quantity (copies/ µL), marked by a corresponding symbol. The *x axis* denotes the cycle number of the two-step QRT-PCR. The *y axis* denotes the ΔRn, which is the fluorescence intensity

over the background. ***(B)***, plot of the threshold cycle (C_T) against the input target quantity (common log scale). The correlation coefficient was 0.99.

(A)



(B)

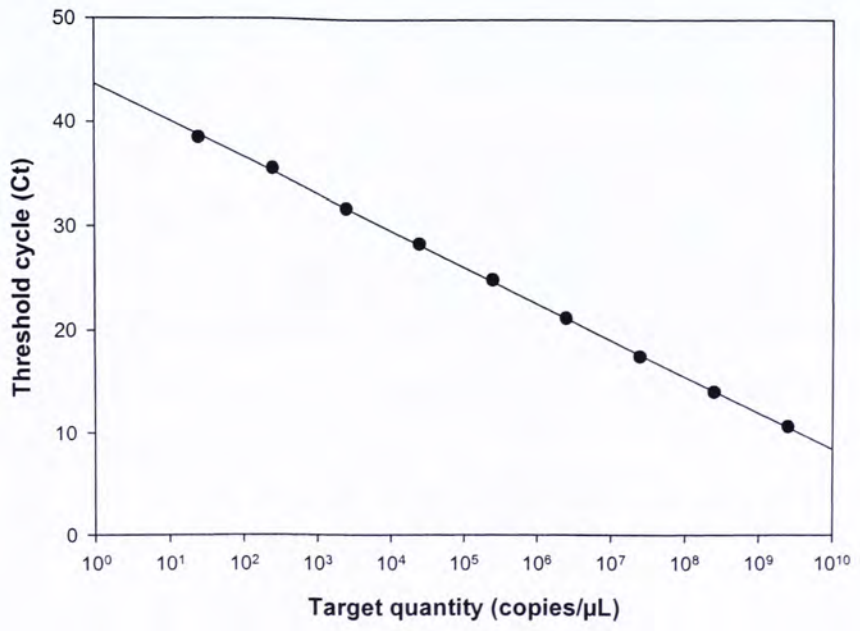


Figure 4.3 Calibration curves of two-step QRT-PCR for miR-16 using RNA and DNA oligonucleotides.

(A), plot of the threshold cycle (C_T) against the input target quantity (common log scale) using RNA oligonucleotide of miR-16 as a calibrator. The correlation coefficient and gradient were 0.99 and -3.55, respectively. ***(B)***, plot of the threshold cycle (C_T) against the input target quantity (common log scale) using DNA oligonucleotide of miR-16 as a calibrator. The correlation coefficient and gradient were 0.99 and -3.56, respectively.

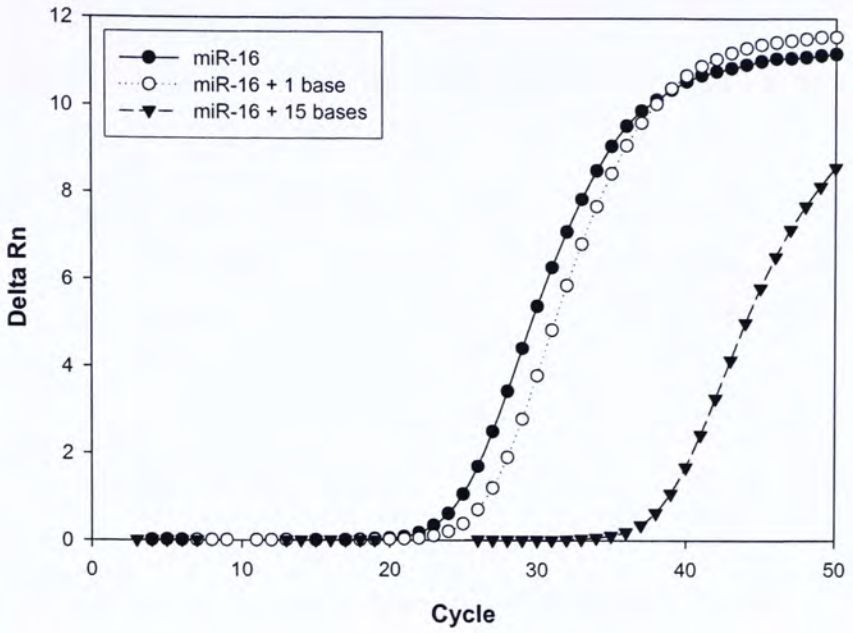


Figure 4.4 Two-step QRT-PCR assays on DNA oligonucleotides coding for miR-16 without any base extension, with 1 base extension and with 15 bases extension at the 3' end.

Amplification plots obtained using two-step QRT-PCR for the 3 DNA oligonucleotides. Each plot corresponds to a particular input oligonucleotide, that code for miR-16, miR-16 + 1 base and miR-16 + 15 bases at the 3'end. Each of the plots is marked by a corresponding symbol. The *x axis* denotes the cycle number of the two-step QRT-PCR. The *y axis* denotes the delta Rn, which is the fluorescence intensity over the background.

Table 4.2 Quantities of miR-141 remained after RNA sample was subjected to different combinations of DNase I and/or RNase A treatments.

	Enzyme treatments		Quantities of miR-141 (copies/ng)
	DNase I	RNase A	
Treatment 1	√	√	3
Treatment 2		√	0
Treatment 3	√		2060
Treatment 4			2254

4.3.2 Detection of cell-free miRNA in maternal plasma

Plasma samples from 20 pregnant women in the third trimester of pregnancy were measured for cell-free miR-16. Cell-free miR-16 was detected in all 20 maternal plasma samples being tested. The median concentration of plasma cell-free miR-16 was 2.7×10^7 copies/mL (interquartile range, 2.4×10^7-6.2×10^7 copies/mL).

To assess the precision of the whole analytical procedure, including the RNA extraction, reverse transcription, and amplification steps, a randomly chosen plasma sample was divided into 18 aliquots for 18 replicated RNA extractions and two-step QRT-PCR assays. The CV% for the C_T value was 1.1 % (Mean±SD, 22.9±0.3) for this analytical system involving RNA extraction and miRNA quantification.

4.4 Discussion

Previously, the detection of cell-free mRNA transcripts in the plasma and serum of human individuals has already opened up many new diagnostic possibilities (Lo and Chiu 2007). Quantitative measurements of circulating placental mRNA transcripts have provided a noninvasive and, at that time, novel approach for the diagnosis of pathological conditions involving the quantitative aberrations of mRNA transcripts expressed in the placenta, such as pre-eclampsia (Ng *et al.* 2003a) and fetal chromosome aneuploidies (Ng *et al.* 2004). In addition, this application of placental-specific mRNA represents a significant step towards the development of universal nucleic acid markers that can be used for all pregnant women, regardless of fetal gender and genetic polymorphisms.

Recently, miRNAs are increasingly being recognized as important regulators of cellular and biological functions. In particular, aberrant expressions of miRNAs are

reported in pathological conditions such as cancers and developmental defects (Bartel 2004). Given the pseudomalignant nature of the human placenta, one may wonder if placental miRNAs would, like DNA and mRNA, be released into maternal plasma. This hypothesis, if proven to be true, may provide a novel class of universal nucleic acid markers that is also not limited by fetal gender and genetic polymorphisms for noninvasive prenatal diagnosis. However, previously, whether miRNAs are present in maternal plasma, and even if they are present, whether they are stable enough for robust detection in human plasma have remained unexplored. In this part of the thesis, I have demonstrated that one miRNA, miR-16, does exist and exists at such a high concentration in maternal plasma that robust extraction and detection can be achieved.

The first part of this study aimed to develop and to evaluate an approach for the quantification of miRNAs. Using the mature miR-16 sequence as a target, I developed a two-step QRT-PCR system from the TaqMan® MicroRNA Assay (Applied Biosystems) which was originally designed for relative quantification of miRNAs. The data demonstrated that the two-step QRT-PCR system could detect over a dynamic range of over 9-log difference with a correlation coefficient of 0.99. The detection by two-step QRT-PCR was highly precise with a CV% for C_T as low as 0.53%. I also demonstrated the specificity of the system. The data showed that the assay could differentiate a target with even a single base extension on its 3' end. It was also capable of targeting miRNA sequences, but not fragmented genomic DNA contamination carried over from the RNA extraction.

In addition, because the TaqMan® MicroRNA Assay (Applied Biosystems) involves the use of rMoMuLV which can reverse transcribe both DNA and RNA

oligonucleotides, I further investigated if both DNA and RNA oligonucleotides were suitable to be used as calibrator to produce similar PCR efficiencies. The use of DNA, instead of RNA, oligonucleotides as calibrators would greatly reduce the cost of the experiments, especially in the subsequent larger-scale experiments as described in Chapter 5. The data revealed similar PCR efficiencies of the RT-PCR assays when using either DNA or RNA oligonucleotides as calibrators. Hence the use of DNA oligonucleotides as calibrators would help reducing cost, by about 6-fold, of the subsequent studies, but maintaining the accuracy of the results.

In the second part of this study, I further sought to evaluate the possibility of plasma miRNA analysis. Using a column-based RNA extraction protocol for RNA extraction (Chapter 3.1) and the two-step QRT-PCR system, I demonstrated, for the first time, that miRNAs could be detected in maternal plasma. MiR-16 was chosen as the target of this initial evaluation because it was found to be a highly and ubiquitously expressed miRNAs, especially in blood cells. I reasoned that such a high level in blood cells, which is in close contact with the plasma, would provide a higher chance of being detected. The median concentration of miR-16 in plasma was 2.7×10^7 copies/mL (interquartile range, 2.4×10^7–6.2×10^7 copies/mL). This analytical system involving both RNA extraction and miRNA quantification was shown to have a CV% for C_T of 1.1 %. Such a high concentration of miR-16 and the highly reliable analysis make plasma miRNA analysis practical for the further development as bio-markers for diagnostic purposes. In particular, because the aberrantly lowered levels of miR-16 were repeatedly reported in the bloods cells of patients with chronic leukemia lymphoma (CLL) (Calin *et al.* 2002, Calin *et al.* 2005), the detection of miR-16 in plasma may have broader implications in the field of CLL diagnosis and monitoring. It would be interesting to compare if miR-16 concentration in the

cell-free portion of plasma might be different compared to that in the leukocytes of these patients. It would also be interesting to see if plasma concentration of miR-16 might have any diagnostic or prognostic potential in CLL. Furthermore, if the aberrant expression of miR-16 can be generalized to other hematological cancers, then a new approach for studying and assessing hematological cancers may be possible.

In this study, to enhance the extraction of small-RNAs, I employed a RNA extraction protocol (Chapter 3.1) based on that developed by Ng *et al.* (2002). The major difference is the use of a column-based RNA isolation protocol of mirVana™ miRNA Isolation Kit (Ambion) in my current protocol. Unlike other RNA extraction columns which were unable to retain small RNA below 200 nucleotides long, the filter cartridge (Ambion) was specially designed for the purification of small RNAs, including miRNAs, which were only about 21-25 bases long. My protocol involved an organic extraction using TRIzol® or TRIzol® LS reagent (Invitrogen) followed by the immobilization of RNA on glass-fiber filters designed for purifying small RNAs using a microspin protocol for the subsequent steps.

In conclusion, this part of the study has described the development and evaluation of a highly accurate and quantitative approach for the analysis of plasma miRNAs. Moreover, I was the first to demonstrate the presence of cell-free miRNAs in maternal plasma. These novel discoveries have further broadened the potential applications of circulating nucleic acids in maternal plasma for noninvasive prenatal diagnosis. However, these results were generated mainly on a miRNA ubiquitously expressed by all human tissues. Further investigations on the possibility of detecting miRNAs released by the placenta into maternal plasma were conducted in Chapter 5.

CHAPTER 5: SYSTEMATIC IDENTIFICATION AND CHARACTERIZATION OF PLACENTAL MICRORNAS IN MATERNAL PLASMA

5.1 Introduction

The successful extraction and detection of circulating cell-free miRNAs in maternal plasma offer new possibilities for noninvasive prenatal investigation. In Chapter 4, it was shown that, with an ubiquitously and highly expressed miRNA, miR-16, circulating cell-free miRNA from plasma could be robustly extracted using a newly devised plasma RNA extraction protocol (Chapter 3.2.1) and be accurately quantified by two-step QRT-PCR assays.

The next step towards developing circulating miRNA as a tool of noninvasive prenatal investigation is to search for placental miRNAs in the plasma of pregnant women using techniques as described in Chapter 4. Previously, the placenta has been shown to release its mRNA transcripts into maternal plasma (Ng *et al.* 2003b). Here, I hypothesized that the placenta would also release its miRNA into maternal plasma, so I systematically studied the levels of 157 miRNAs, the miRNA assays of which were available in the TaqMan® MicroRNA Assays Early Access Kit – Human Panel (Applied Biosystems), in the placenta. Furthermore, in another previous study, the hematopoietic compartment was shown to be the predominant source of circulating DNA in the plasma of non-pregnant individuals (Lui *et al.* 2002). Here, I also hypothesized that the maternal blood cells would be the predominant source of non-placental miRNA in maternal plasma. So I studied and compared the miRNA profile from maternal blood cells, and selected only those miRNAs that were

expressed in the placenta at much higher levels than in maternal blood cells for further analysis. Ideally, fetal-derived markers in maternal plasma should be cleared after delivery of the fetus. So I further narrowed down my systematic search to include only those miRNAs that were not detectable in maternal plasma collected 24 hours after delivery (Tsui *et al.* 2004).

Novel miRNAs are being discovered continuously. In May 2007, 475 mature miRNA sequences were recorded in the Sanger Center MicroRNA Registry, Release 9.2 (Griffiths-Jones *et al.* 2006). Since the functions of most miRNAs remain unexplored, it would be difficult to use a "candidate transcript" approach, as in the discovery of circulating fetal mRNAs (Ng *et al.* 2003b). Instead, I systematically screened for placental miRNAs in maternal plasma using the two-step QRT-PCR assays with 157 assays available in the TaqMan® MicroRNA Assays Early Access Kit – Human Panel (Applied Biosystems). This strategy would systematically identify placental miRNAs detectable in maternal plasma. I hope that the results of this study can provide quantitative information for evaluating the potential use of placental miRNAs in maternal plasma for noninvasive diagnostic tests of pregnancy-associated disorders.

This part of the thesis aims to search for and to characterize placental miRNAs in maternal plasma for future use in prenatal diagnosis. Firstly, using TaqMan® MicroRNA Assays, I systematically screened for placental miRNAs in maternal plasma. Secondly, I sought to decipher the biological properties, such as the temporal profile of placental miRNA concentration in maternal plasma during the three trimesters of pregnancy, and the physical properties, such as the possible association with particulate matter and stabilities of purified miRNA compared to purified mRNA in plasma. Such fundamental information on placental miRNA in maternal

plasma would form the ground work to further develop them into noninvasive biomarkers for prenatal monitoring.

5.2 Materials and methods

5.2.1 Sample collection

Peripheral blood samples were collected from pregnant women, with singleton, uncomplicated pregnancies, just before and at 24 hours after elective caesarean delivery. Paired term placental tissues were also collected just after the delivery. For the module studying the temporal profile of placental miRNA concentration in maternal plasma, peripheral blood samples were collected from 10 first-trimester (gestational age range: 12–14 weeks), 10 second-trimester (gestational age range: 17–19 weeks), and 10 third-trimester (gestational age range: 37–39 weeks) pregnancies. In addition, peripheral blood samples were collected from a healthy male with informed consent to test the stability of purified placental mRNA and miRNA in plasma (Chapter 5.2.2). All the samples were processed using the protocol described in Chapter 3.1. All pregnant women were recruited with informed consent for the Department of Obstetrics and Gynecology, Prince of Wales Hospital, Shatin, Hong Kong. The study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee.

5.2.2 Experimental design

Identification of placental miRNAs in maternal plasma

In the first part of this study, I sought to identify cell-free placental miRNAs in maternal plasma from a panel of 157 miRNAs. The 157 assays were supplied as the TaqMan® MicroRNA Assays Early Access Kit – Human Panel (Applied Biosystems).

In brief, paired samples of pre-delivery maternal plasma and blood cells, post-delivery maternal plasma and placental tissue from five healthy pregnant women were tested for the concentrations of each of the 157 miRNA targets using two-step QRT-PCR assays as described in Chapter 3.3.3.3. The standard curve was constructed using serial dilutions of synthetic DNA oligonucleotide (Proligo) on each target miRNA with concentrations ranging between 6.25 x 10^6 and 62.5 copes per RT reaction. By using a systematic screening strategy outlined in Figure 5.1, placental miRNA in maternal plasma were identified.

Detection rates and clearance kinetics of placental miRNAs in maternal plasma

Detection rates, which are the percentages of the cases with detectable amount of the respective miRNAs, and clearance kinetics of placental miRNAs, identified as potentially detectable in maternal plasma in the previous part, were further investigated. Since a smaller number of miRNAs were to be investigated in detail, I studied them using more concentrated RNA preparations extracted from the same volume of maternal plasma. In this part, total RNA extracted from 1.6 mL of plasma were eluted into 20.35 μL RNA solution. In contrast, in the screening of the previous part, the extracted total RNA was further diluted to 132 μL, so that more miRNA assays could be performed initially. Paired pre- and 24-hour post-delivery maternal plasma from 10 healthy pregnant women (gestational age, 37–40 weeks) were measured for the concentrations of each of the candidate miRNA targets using two-step QRT-PCR systems as described in Chapter 3.3.3.3.

Investigation on the effects of filtration on the concentrations of placental miRNA and mRNA transcript in maternal plasma

Since the placental miR-141 was most readily detectable in maternal plasma with the

highest detection rate and concentrations among the miRNAs investigated, it was chosen for further characterization. To study if this circulating placental miR-141 and *hPL* mRNA were particle-associated, I passed maternal plasma through filters of different pore sizes, and quantifying the concentrations of miR-141 and *hPL* mRNA in the filtrate. Plasma samples of 15 healthy women in their third trimester of pregnancies (gestational age, 38–40 weeks) were collected. Each plasma sample was divided into four aliquots: three were individually passed through filters (Millex-GV, Millipore, MA) with pore sizes of 0.22, 0.45 and 5 μm. The remaining aliquot was not subjected to any filtration. The miR-141 concentration in each of the processed sample was measured using two-step QRT-PCR assays as described in Chapter 3.3.3.3.

Stability of purified placental miRNA and RNA in plasma

The stability of exogenously added, purified, placental miRNA and mRNA in plasma was studied and compared. Two hundred and twelve nanograms of placental RNA, extracted as described in Chapter 3.2.1, were added to each of the six 0.8-mL aliquot of plasma obtained from a randomly selected, healthy, male human subject and were incubated for 0 s, 5 s, 15 s, 60 s, 1 hr, and 2 hr at room temperature. A control plasma sample without RNA added was included. After incubation, 1 mL of TRIzol® LS reagent (Invitrogen) was immediately added to stop the ribonuclease activity in blood. The plasma samples were measured for *hPL* mRNA and miR-141 concentrations with QRT-PCR as described in Chapter 3.3.2.2 and 3.3.3.3, respectively. The amounts of mRNA and miR-141 remaining in the plasma after the incubation were expressed as percentages relative to their corresponding starting concentrations at 0-s incubation time.

Temporal profile of circulating placental miRNA in maternal plasma during pregnancy

To study the gestational variation of the plasma concentration of the placental miR-141, peripheral blood samples were obtained from 30 pregnant women at various stages of gestation, in which 10 women were at the first trimester (gestational age, 12–14 weeks), 10 women were at the second trimester (gestational age, 17–19 weeks) and 10 women were at the third trimester (gestational age, 37–39 weeks). The miR-141 concentration in each of the processed sample was measured using the two-step QRT-PCR assay as described in Chapter 3.3.3.3.

5.2.3 RNA extraction and miRNA quantification

Processing of peripheral blood samples and placental tissue for cell-free plasma followed procedure as described in Chapter 3.1. Two-step QRT-PCR assays for miRNAs quantification followed the procedures as described in Chapter 3.3.3.3. TaqMan® MicroRNA Assays of miRNAs were ordered from Applied Biosystems. Serial dilutions of synthetic oligonucleotides were used for the absolute quantifications of the miRNAs.

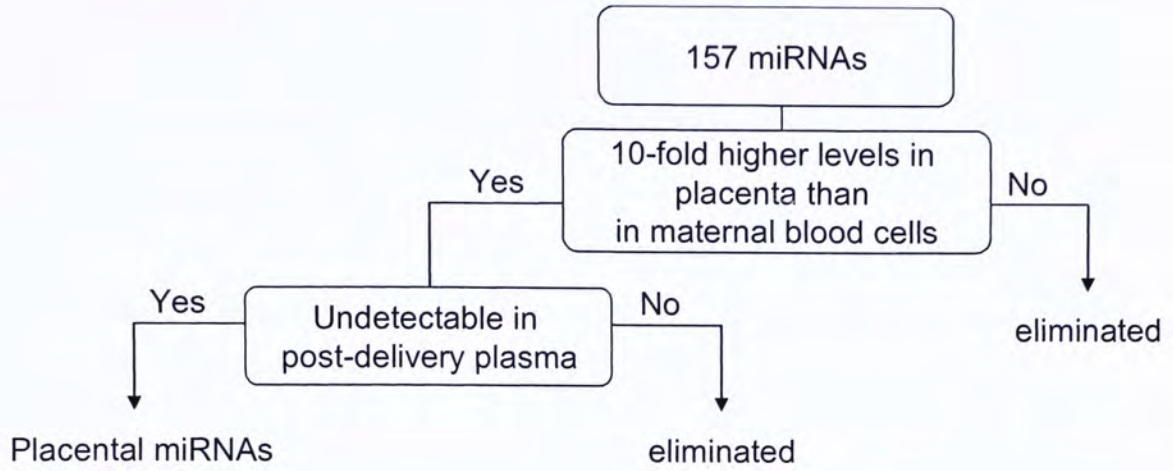


Figure 5.1 Schematic diagram of the strategy used for the systematic identification of placental miRNAs in maternal plasma.

Paired placental tissues and maternal blood cell samples were collected and subjected to two-step QRT-PCR assays. MiRNAs expressed in the placenta at 10-fold or higher levels than in maternal blood cells were selected. Paired pre- and post-delivery plasma samples were collected and subjected to the two-step QRT-PCR assays. Since I aimed to identify candidate miRNA markers in plasma for pregnancy, the miRNAs that were not detectable in post-delivery maternal plasma were listed in bold in Table 5.1.

5.3 Results

5.3.1 A systematic search for placental miRNAs in maternal plasma using two-step QRT-PCR assays

In this study, I employed a strategy as outlined in Figure 5.1 to identify candidate miRNA markers in maternal plasma. The levels of each candidate miRNAs in five cases of paired sample of pre-delivery plasma, 24-hour post-delivery plasma, placental tissue and maternal blood cells were obtained by the two-step QRT-PCR assay, as described in Chapter 3.3.3, for analysis.

Previously, Lui *et al.* (2002) showed that the hematopoietic compartment was the predominant source of circulating DNA in the plasma of non-pregnant individuals. Based on this observation, Tsui *et al.* (2004) further hypothesized that much of the background maternal mRNA in maternal plasma also originated from the hematopoietic compartment, while the fetal mRNA mainly originated from the placental tissue. Here, I hypothesized that miRNAs expressed in the placenta would also give rise to the fetal-derived miRNAs amongst the background of maternal blood cells-dervied miRNAs in the maternal plasma. Because the aim of this part of the thesis is to identify placental miRNAs in maternal plasma, I systematically identified candidate miRNAs that have much higher levels in placental tissue than in paired maternal blood cells using 157 miRNA assays. Thus, 34 miRNAs that were expressed with at least 10-fold higher levels in placental tissue than in maternal blood cells were shortlisted (Table 5.1). Moreover, using the sensitive and quantitative QRT-PCR assays and with the reasonably high concentration of miRNAs, quantitative data on the presence of these target miRNA in pre- and 24-hour post-delivery plasma were also generated. Among them, 17 miRNAs were further

shortlist because they were completely undetectable in any 24-hour post-delivery maternal plasma samples collected from the five pregnancies. This panel represents the placental miRNAs that can be potentially developed as markers in maternal plasma for the noninvasive monitoring of pregnancy.

Table 5.1 List of placental miRNAs that can be potentially developed as markers in maternal plasma for the noninvasive monitoring of pregnancy (Chapter 5.3.1).

	Median concentration (copies/ng)			Detection rate (%) [d]	
	Placenta	Maternal blood cells	Fold change[e]	Pre-delivery plasma	Post-delivery plasma
miR-hsa-125b	821926	9778	84.1	100	100
miR-hsa -141 #	**760118**	**6565**	**115.8**	**40**	**0**
miR-hsa-125a	539358	7852	68.7	100	100
miR-hsa-100	339088	5794	58.5	100	100
miR-hsa-99a	208557	3314	62.9	80	80
miR-hsa-200c	178648	2365	75.5	100	40
miR-hsa-205	39828	0[$]	N/A	100	60
miR-hsa-135b #	**34684**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-224	20908	1235	16.9	60	80
miR-hsa-149 #	**14986**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-127	13630	641	21.3	20	40
miR-hsa-299-5p #	**11452**	**359**	**31.9**	**20**	**0**
miR-hsa-154* #	**10904**	**559**	**19.5**	**0**	**0**
miR-hsa-34c	**9145**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-214	9086	48	189.3	60	100
miR-hsa-34a	8679	808	10.7	40	80
miR-hsa-133a	6084	386	15.8	20	80
miR-hsa-218	5723	381	15.0	0	40
miR-hsa-200b	**4088**	**392**	**10.4**	**0**	**0**
miR-hsa-139	**3919**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-133b	3891	290	13.4	100	100
miR-hsa-154	**3408**	**207**	**16.5**	**0**	**0**
miR-hsa-203	3404	0[$]	N/A	20	20
miR-hsa-323	2675	121	22.1	20	20

miR-hsa-368	**1895**	**84**	**22.6**	**0**	**0**
miR-hsa-373	**1895**	**73**	**26.0**	**0**	**0**
miR-hsa-137	**1871**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-184	**1771**	**109**	**16.2**	**0**	**0**
miR-hsa-372	**957**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-371	**412**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-211	379	0[$]	N/A	20	20
miR-hsa-34b	**332**	**30**	**11.1**	**0**	**0**
miR-hsa-337	**84**	**0[$]**	**N/A**	**0**	**0**
miR-hsa-198	37	0[$]	N/A	20	20

[a] Names of the 17 microRNAs which were undetectable in 24-hour post-delivery plasma are shown in bold.
[b] Placental microRNAs chosen for further characterization in maternal plasma were marked with ([#]). Concentrations of samples without amplification signal detected were referred to as 0 copies/mL or 0 copies/ng ([$]).
[c] MiRNAs are sorted in descending order of the median concentration in the placenta.
[d] Detection rate is the percentage of the number of cases with detectable amount of the respective miRNA.
[e] Fold change is the ratio of the concentration of a miRNA in the placenta to that in the maternal blood cells.

5.3.2 Detection rate and clearance kinetics of placental miRNAs in maternal plasma

The detection rates, which are the percentages of the cases with detectable amount of the respective miRNAs, and clearance kinetics of some of the candidate miRNA markers for pregnancy in maternal plasma were further analyzed. Among the panel identified systematically in the previous section, the five miRNAs most highly expressed in the placenta and undetectable in any of the five post-delivery maternal plasma samples were chosen for further investigation. The reason behind this choice was based on our previous experience with placental mRNA transcripts in maternal plasma. Those mRNA transcripts that were most highly expressed in the placenta were also most readily detectable in maternal plasma. As an extrapolation of this concept, I presumed the most highly expressed miRNA in the placenta would also be the most readily detectable in maternal plasma.

Among the 10 pregnant women recruited for this part of the study, miR-141 was 100% detectable in the pre-delivery plasma but only in 50% detectable in the 24-hour post-delivery plasma, with median concentrations of 4391 (interquartile range: 1576-8279 copies/mL) and 241 copies/mL (interquartile range: 0-1323 copies/mL), respectively (Figure 5.2A). A statistically significant decrease in maternal plasma miR-141 concentration was observed before and after delivery (Wilcoxon test, $P = 0.003$). MiR-135b was 20% detectable in the pre-delivery plasma but undetectable in any 24-hour post-delivery plasma, with the median concentration of 0 (interquartile range: 0-0 copies/mL) in the pre-delivery plasma (Figure 5.2B). No systematic alteration in maternal plasma miR-135b concentration was observed before and after delivery (Wilcoxon test, $P = 0.5$). MiR-149 was 80% detectable in the pre-delivery

plasma but undetectable in any 24-hour post-delivery plasma, with the median concentration of 594 (interquartile range: 336-893 copies/mL) in the pre-delivery plasma (Figure 5.2C). A statistically significant decrease in maternal plasma miR-149 concentration was observed before and after delivery (Wilcoxon test, P = 0.003). MiR-299-5p was 50% detectable in the pre-delivery plasma but only 20% detectable in the 24-hour post-delivery plasma, with median concentrations of 225 (interquartile range: 0-553 copies/mL) and 0 copies/mL (interquartile range: 0-0 copies/mL), respectively (Figure 5.2D). No systematic alteration in maternal plasma miR-299-5p concentration was observed before and after delivery (Wilcoxon test, P = 0.063). MiR-154* was undetectable in any pre-delivery plasma and 24-hour post-delivery plasma even with the more concentrated plasma RNA input (Figure 5.2E). As a control, detection of miR-29a was performed alongside with each sample to show that the extraction methods worked well for all samples. MiR-29a was chosen because it was detected at similarly high levels in both pre- and post-delivery plasma. MiR-29a was detected in all pre- and 24-hour post-delivery plasma samples with median concentrations of 40559 (interquartile range: 27178-50614 copies/mL) and 34667 copies/mL (interquartile range: 31018-40594 copies/mL), respectively (Figure 5.2F). No systematic alteration in maternal plasma miR-29a concentration was observed (Wilcoxon test, P = 0.274), as expected for such control miRNA.

In summary, 4 out of 5 of the placental miRNA investigated in this part were detected in maternal plasma at higher detection rates and at higher median concentrations before delivery, compared to those after deliveries. It showed their association with pregnancy and thus they were potentially applicable for use in prenatal monitoring.

The low detection rates of miR-135b and miR-299-5p and miR-154* indicated that there were still rooms for improvement in the miRNA extraction and miRNA quantification.

5.3.3 Effects of filtering maternal plasma on the concentration of placental miRNA and mRNA

Since miR-141 was the most readily detectable in maternal plasma, I sought to further characterize its nature. To decipher the physical nature of this placental miRNA in maternal plasma, I filtered 0.8 mL aliquots of maternal plasma from 15 pregnant women through filters with pore sizes of 5 μm, 0.45 μm and 0.22 μm, quantified the concentration of this placental miR-141 in the filtrate, and compared with that without filtration. No statistically significant difference was found in the concentration of miR-141 among plasma samples filtered through different-sized pores (Friedman test, $P = 0.257$, Figure 5.3A). The median concentrations of miR-141 were 6082 copies/mL (interquartile range: 4211-8803 copies/mL), 7317 copies/mL (interquartile range: 5669-11087 copies/mL), 6115 copies/mL (interquartile range: 4779-10264 copies/mL), and 5472 copies/mL (interquartile range: 4536-10123 copies/mL) in plasma unfiltered, filtered through 5-, 0.45-, and 0.22-μm filters, respectively. Hence, these data suggested that placental miR-141 in maternal plasma was not associated with subcellular particles > 0.22-μm in diameter.

In contrast to these miR-141 results, with the same experiment, the median concentrations of *hPL* mRNA were 980 copies/mL (interquartile range: 536-2193 copies/mL), 834 copies/mL (interquartile range: 0-1226 copies/mL), 0 copy/mL (interquartile range: 0-0 copy/mL), and 0 copy/mL (interquartile range: 0-605 copies/mL) in plasma unfiltered, filtered through 5-, 0.45-, and 0.22-μm filters,

respectively. These data show that filtration caused a clearly observable reduction in *hPL* mRNA concentrations in plasma samples (Friedman test, $P < 0.05$, Figure 5.3B). Pair-wise analysis further indicated that statistically significant differences were detected in every pair of these filter sizes (Student-Newman-Keuls Method, $P < 0.05$ for each pair). In paired plasma samples with detectable *hPL* concentration, the median concentration was reduced by 1.82-fold as the plasma sample was passed through 5 μm-filters, and was further reduced by 1.69-fold as they were passed through 0.45 μm-filters (Table 5.2). The plasma *hPL* mRNA concentration decreased to undetectable level after the plasma had been filtered through the 0.22 μm filters. This is consistent with the previously published results on the particle-associated nature of *hPL* mRNA in maternal plasma (Tsui *et al.* 2002).

Table 5.2 Folds of reduction in *hPL* mRNA concentrations when plasma was passed through filters of different pore sizes.

	Filtered samples (filter pore size)			Overall reduction
	5 μm	0.45 μm	0.22 μm	
***hPL* mRNA**	1.82-fold	1.69-fold	-	-

The reduction-fold was calculated by dividing the mRNA level of a sample passing through a particular size filter, by the mRNA levels of the same sample passing through a filter in the next (bigger) size category.
For paired samples with detectable concentration of *hPL*, the median of the folds of reduction was shown.

5.3.4 Stability of purified placental mRNA and miRNA in plasma

It was surprising that miRNA, being RNA in nature, existed in plasma at reasonably high concentration seemingly without any protection, such as association with subcellular particles, from RNase activities in blood. Intrigued by this surprising observation, I proposed that miRNA might be intrinsically more stable than mRNA transcripts in its purified, naked form, without any association with particles or proteins, in plasma. To explore this proposed idea, I exogenously added purified RNA sample, extracted as described in Chapter 3.2.1, into the plasma sample from a randomly chosen healthy male, incubated for different time points, and quantified for the concentration of miR-141 and *hPL* mRNA remaining after incubation. Without any exogenously added placental RNA, there was no detectable concentration of miR-141 and *hPL* in the male plasma as expected (Figure 5.4B). With the addition of purified placental RNA, within the first 15 s, the miR-141 concentration decreased by 24-fold from 100% (1.3×10^6 copies/mL) to 4.1% (5.5×10^4 copies/mL) relative to its starting concentration at 0-s incubation (Figure 5.4B). With a 60-s or up to 2-h incubation, the concentration of miR-141 was observed to remain at about 1% (about 10,000 copies/mL). In contrast, within the first 15 s, *hPL* mRNA transcript was shown to decrease more dramatically for 333-fold from 100.0% (5.5×10^5 copies/mL) to 0.3% (1539 copies/mL) relative to its starting concentration at 0-s incubation (Figure 5.4B). With a 60-s or longer incubation, no *hPL* mRNA could be amplified. This experiment was repeated with lower and higher amounts of incubated placental RNA (106ng, 425 ng, 825 ng) to ensure that such effect was not dependent on the amount of the exogenously added RNA, and similar results were obtained for both miR-141 and hPL (Figure 5.4A, C and D).

In summary, purified miR-141 was degraded at a much slower rate than that of

purified *hPL* mRNA in plasma in the first 15 s. Furthermore, purified miR-141, in the absence of any association with subcellular particles or proteins, still remained detectable in plasma for over 2 hours.

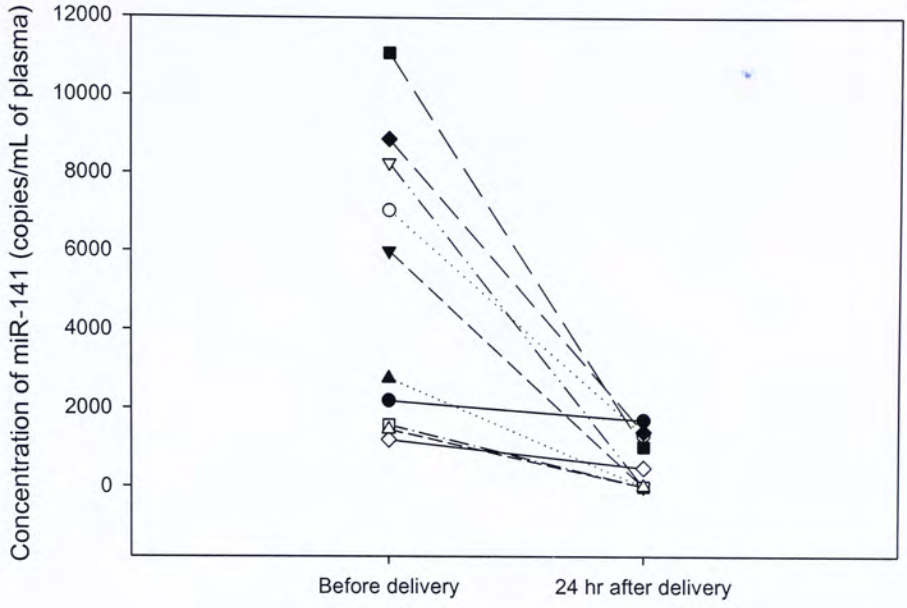
5.3.5 Temporal profile of placental miRNA concentrations in maternal plasma across different trimesters of pregnancies

The placental miR-141 was measured in the plasma of 10 pregnant women each recruited from the first, second and third trimesters of pregnancy (Table 5.3). MiR-141 was 80% detectable in maternal plasma of the first-trimester and 100% detectable in maternal plasma of both the second and the third trimesters. The median miR-141 concentrations were 7756 copies/mL (interquartile range: 4065-9959 copies/mL), 8761 copies/mL (interquartile range: 5378-17090 copies/mL), and 19464 copies/mL (interquartile range: 18240-29852 copies/mL) for the first-, second-, and third-trimester maternal plasma, respectively. Overall, circulating miR-141 concentration showed an increasing trend with gestational age (Spearman correlation analysis, $r = 0.58$, $P = 0.00$, Figure 5.5).

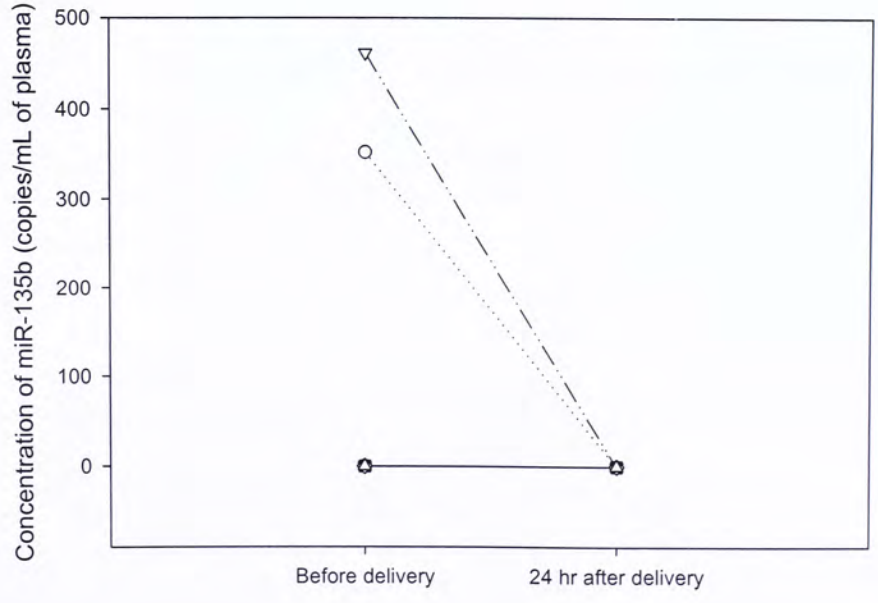
Table 5.3 Concentration of miR-141 in the plasma of pregnant women during the first, second, and third trimesters.

Trimester of pregnancy	miR-141	
	Detection rate	median concentration (copies/mL) (interquartile range)
First	80% (8/10)	7,756 (4,065-9,959)
Second	100% (10/10)	8,761 (5,378-17,090)
Third	100% (10/10)	19,464 (18,240-29,852)

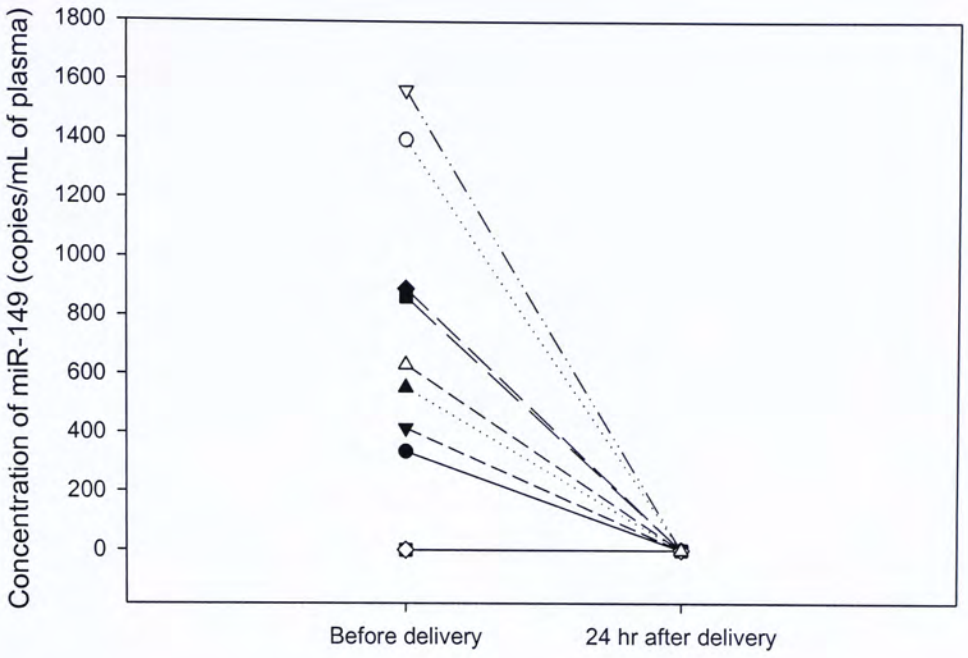
(A)


Concentration of miR-141 (copies/mL of plasma)
12000
10000
8000
6000
4000
2000
0
Before delivery
24 hr after delivery

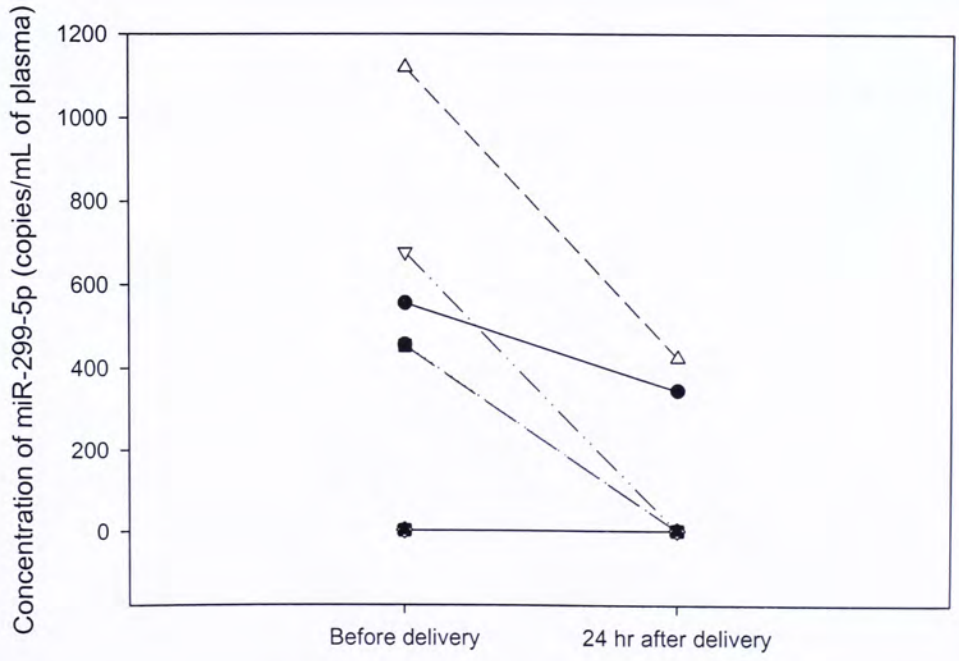
(B)


Concentration of miR-135b (copies/mL of plasma)
500
400
300
200
100
0
Before delivery
24 hr after delivery

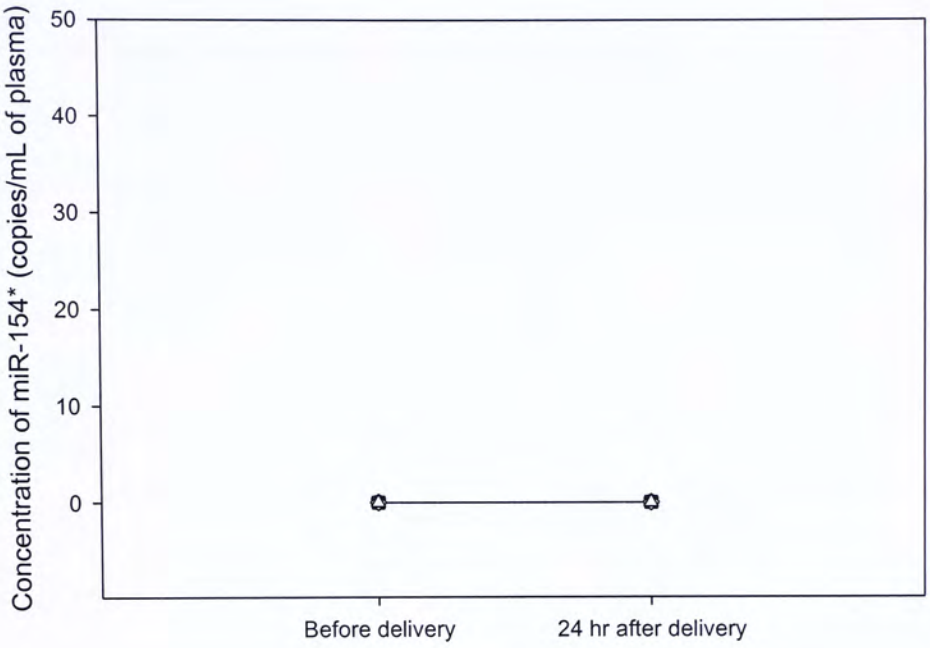
(C)


Concentration of miR-149 (copies/mL of plasma)
1800
1600
1400
1200
1000
800
600
400
200
0
Before delivery
24 hr after delivery

(D)


Concentration of miR-299-5p (copies/mL of plasma)
1200
1000
800
600
400
200
0
Before delivery
24 hr after delivery

(E)


Concentration of miR-154* (copies/mL of plasma)
50
40
30
20
10
0
Before delivery
24 hr after delivery

(F)

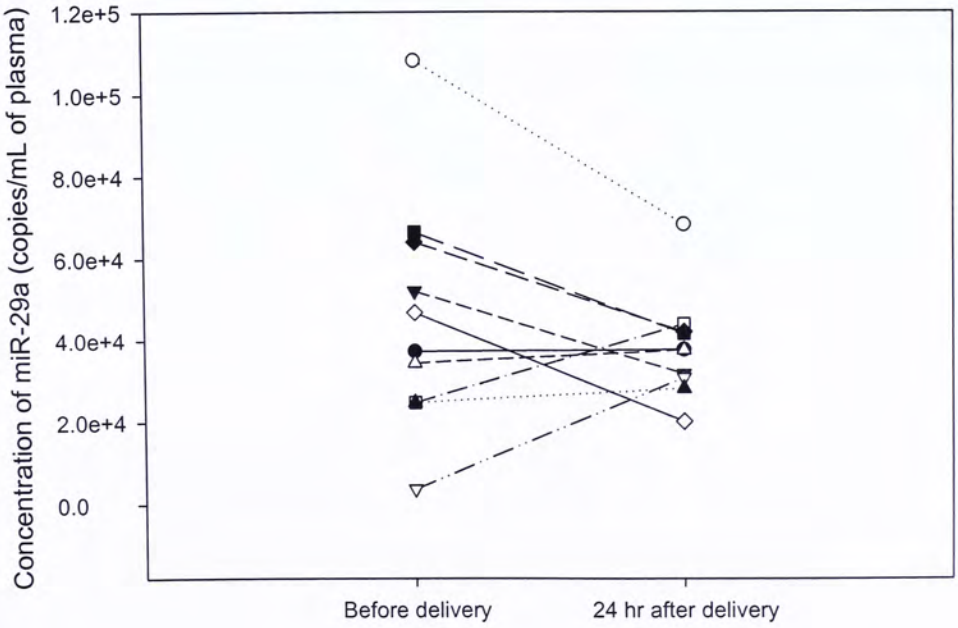
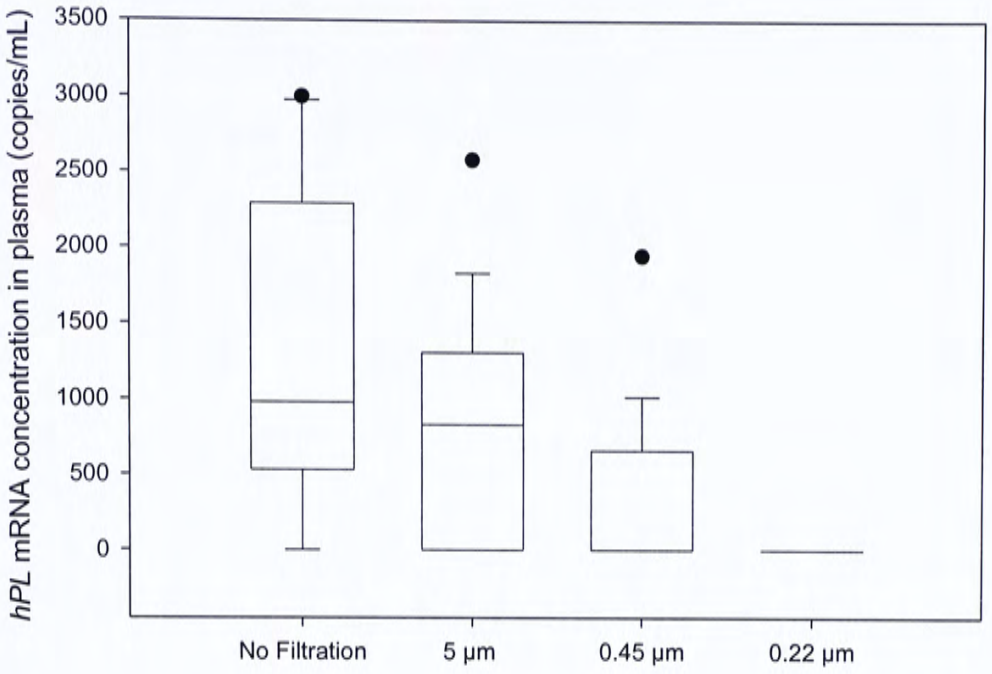

Concentration of miR-29a (copies/mL of plasma)
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Before delivery
24 hr after delivery

Figure 5.2 Clearance kinetics of candidate miRNA markers in maternal plasma for monitoring pregnancy.

Concentrations of ***(A)*** miR-141, ***(B)*** miR-135b, ***(C)*** miR-149, ***(D)*** miR-299-5p, ***(E)*** miR-154*, and ***(F)*** miR-29a in maternal plasma before delivery and at 24 hr after delivery were quantified by the two-step QRT-PCR assays. Each line represents one plasma sample obtained from one subject.

(A)


hPL mRNA concentration in plasma (copies/mL)
3500
3000
2500
2000
1500
1000
500
0
No Filtration
5 µm
0.45 µm
0.22 µm

(B)

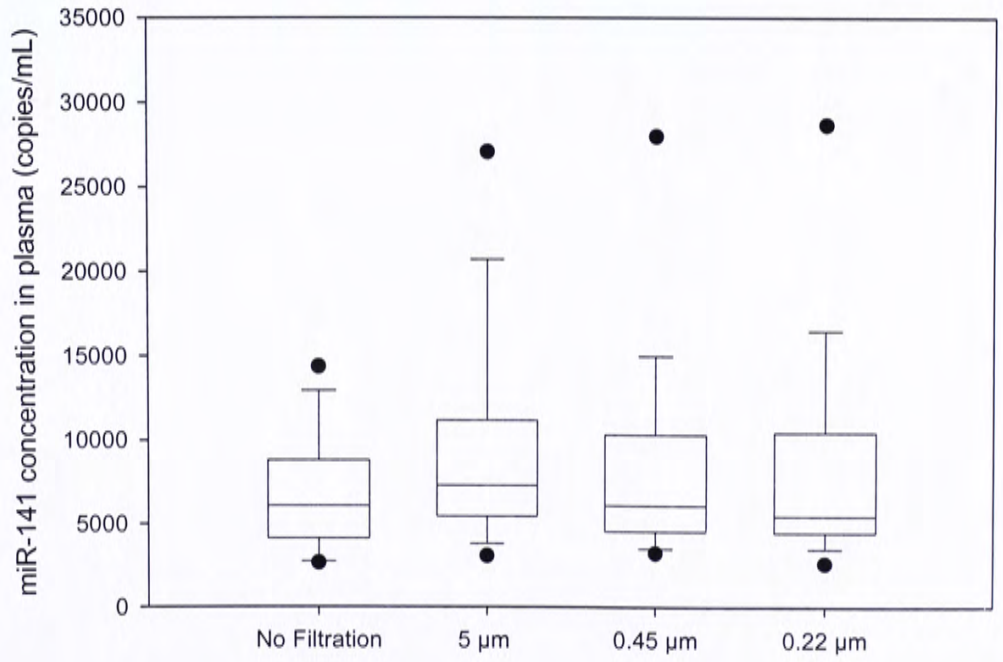
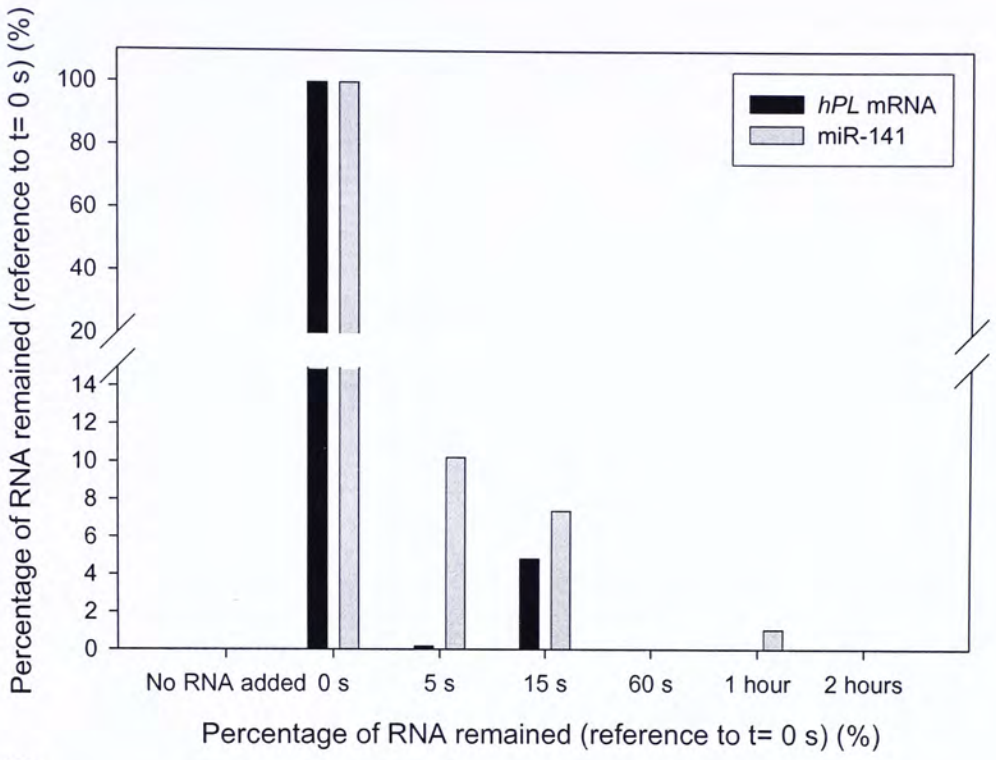

miR-141 concentration in plasma (copies/mL)
35000
30000
25000
20000
15000
10000
5000
0
No Filtration
5 µm
0.45 µm
0.22 µm

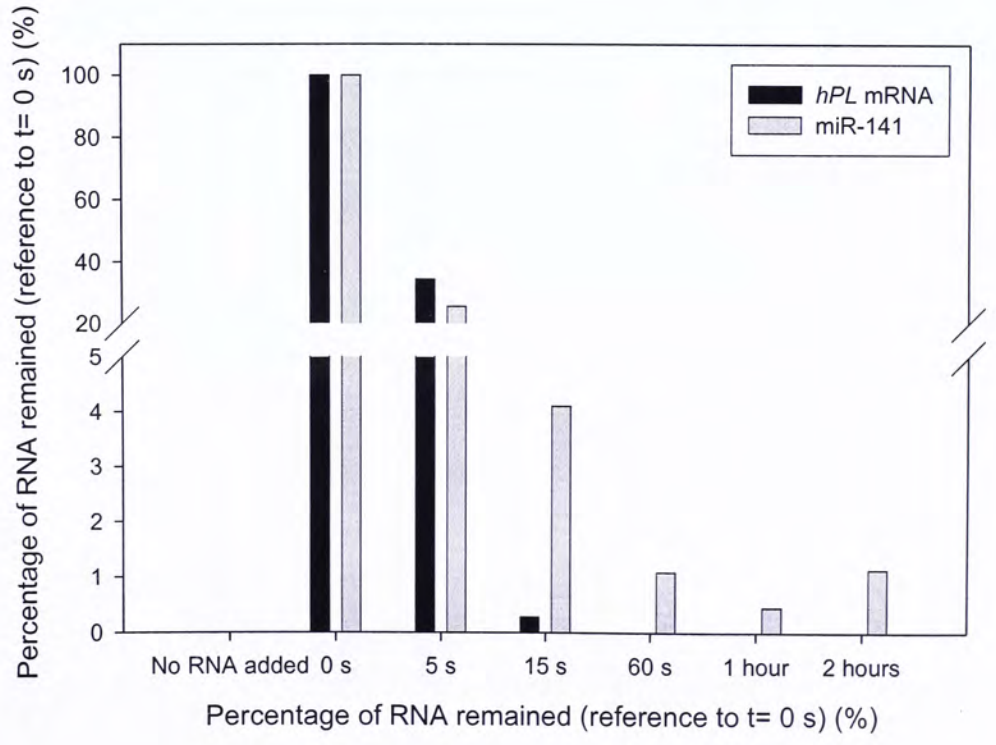
Figure 5.3 Concentrations of placental miR-141 and *hPL* mRNA transcripts in maternal plasma after filtration through filters of different pore sizes.

(A), plasma *hPL* mRNA concentrations (copies/mL), as determined by QRT-PCR *(y axis)*, and ***(B)***, plasma miR–141 concentrations (copies/mL), as determined by QPCR (*y axis*), were plotted against filter pore sizes (*x axis*). The *lines inside the boxes* denote medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicate data points outside the 10th and 90th percentiles. (N.B. Data are presented in the same format for all of the box plots in this thesis.)

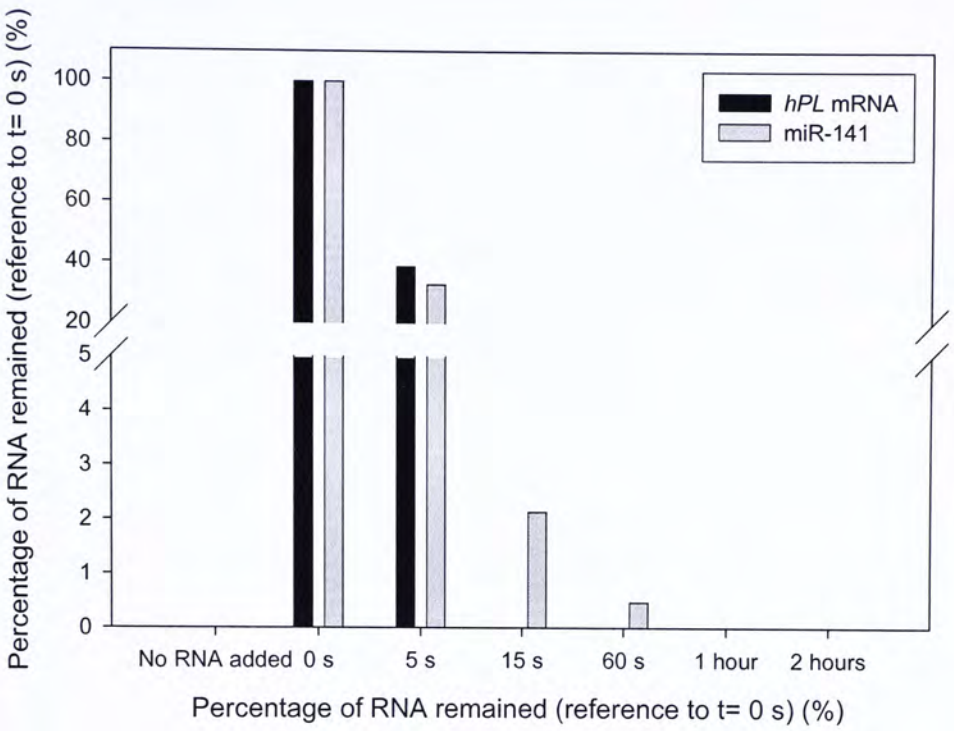
(A)


Percentage of RNA remained (reference to t= 0 s) (%)
100
80
60
40
20
14
12
10
8
6
4
2
0
hPL mRNA
miR-141
No RNA added
0 s
5 s
15 s
60 s
1 hour
2 hours
Percentage of RNA remained (reference to t= 0 s) (%)

(B)


Percentage of RNA remained (reference to t= 0 s) (%)
100
80
60
40
20
5
4
3
2
1
0
hPL mRNA
miR-141
No RNA added
0 s
5 s
15 s
60 s
1 hour
2 hours
Percentage of RNA remained (reference to t= 0 s) (%)

(C)


Percentage of RNA remained (reference to t= 0 s) (%)
100
80
60
40
20
5
4
3
2
1
0
hPL mRNA
miR-141
No RNA added
0 s
5 s
15 s
60 s
1 hour
2 hours
Percentage of RNA remained (reference to t= 0 s) (%)

(D)

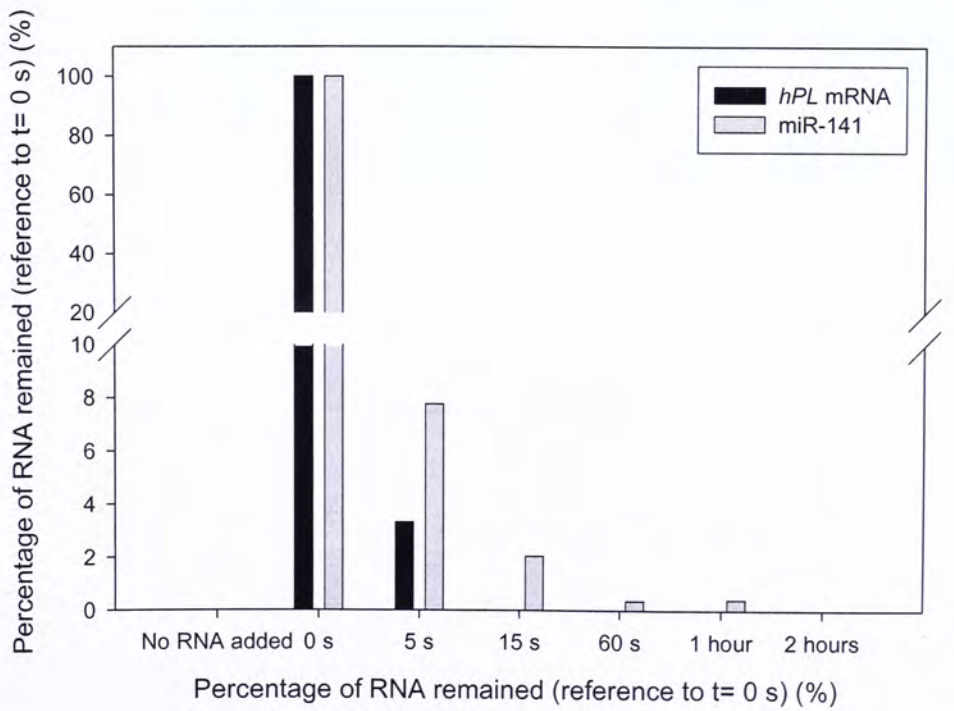

Percentage of RNA remained (reference to t= 0 s) (%)
100
80
60
40
20
10
8
6
4
2
0
hPL mRNA
miR-141
No RNA added
0 s
5 s
15 s
60 s
1 hour
2 hours
Percentage of RNA remained (reference to t= 0 s) (%)

Figure 5.4 Stabilities of purified, exogenously added, placental miRNA and mRNA transcripts in plasma.

Percentage of *hPL* mRNA and miR-141, relative to their respective starting concentrations, remaining in plasma after incubation as determined by QRT-PCR assays are plotted on *y axis*. The incubation time are shown on the *x axis*. ***(A)***, ***(B)***, ***(C)***, and ***(D)*** are graphs correspond to the incubation of 106 ng, 212 ng, 425 ng, and 825 ng, respectively, of total RNA being incubated.

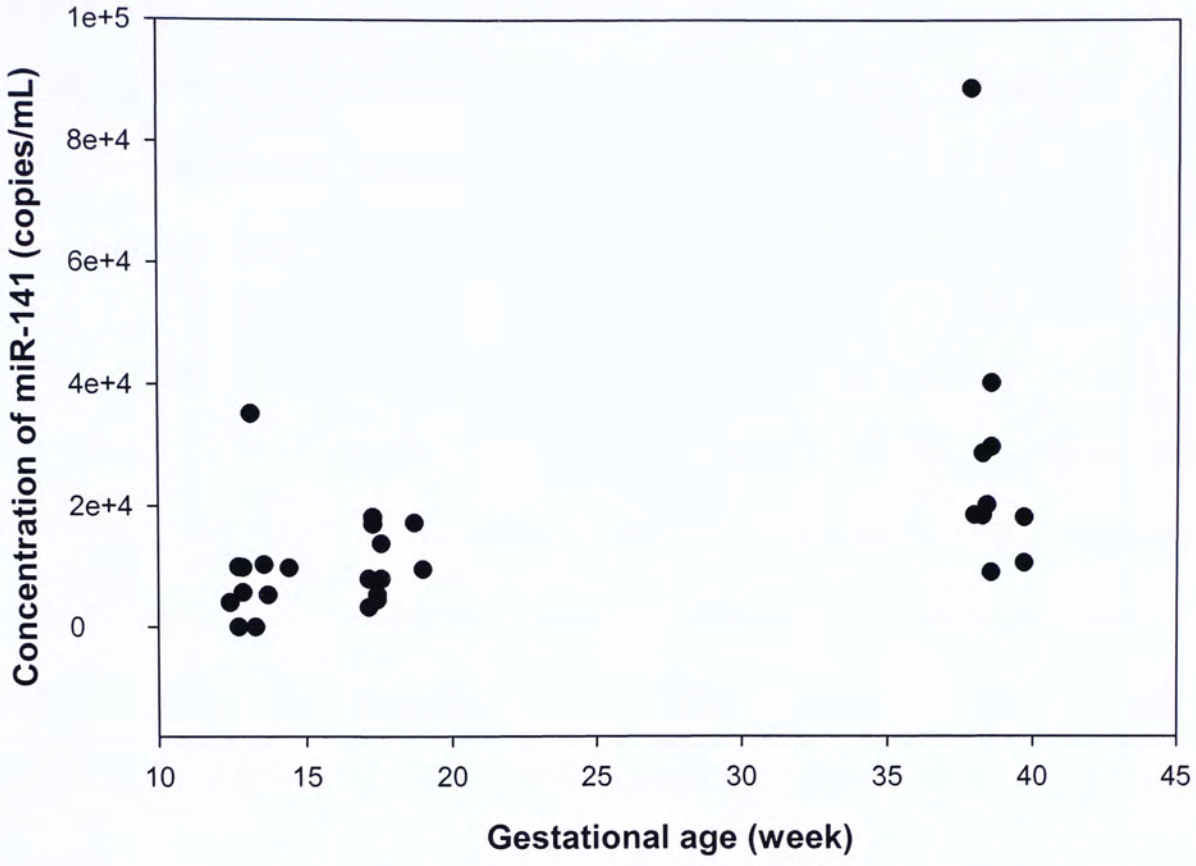


Figure 5.5 Concentration of placental miR-141 in maternal plasma against gestational age.

Concentration of miR-141 (copies/mL) in maternal plasma was shown to be correlated with the gestational age (weeks).

5.4 Discussion

Chapter 4 showed that miRNAs do exist in the cell-free portion of plasma, and can be stably extracted and accurately quantified. Thus, these results have opened up opportunities for using miRNA in plasma as noninvasive markers.

In the first part of this Chapter, I provided the first evidence that placental miRNAs can be detected in the maternal plasma. Data presented in this Chapter supports this hypothesis. Here, I screened for placental miRNAs in maternal plasma from a panel of miRNAs using the strategy as outlined in Figure 5.1. This strategy is based on previous findings that demonstrated the predominant hematopoietic origin of plasma DNA in normal individuals (Lui *et al.* 2002) and that the placenta is a major source circulating fetal mRNA in maternal plasma during pregnancy (Ng *et al.* 2003b). Using two-step QRT-PCR assays for miRNAs quantification, I quantified the levels of each of the miRNAs available in the TaqMan® MicroRNA Assays Early Access Kit – Human Panel (Applied Biosystems). Out of the 157 miRNAs that were screened in this study, 34 of them had at least ten-fold higher levels in placenta than in maternal blood cells. Moreover, using highly sensitive and quantitative QRT-PCR assays and with the extremely high biological levels of miRNA, 17 miRNAs were further shortlisted because they were completely undetectable in 24-hour post-delivery plasma in their initial detections in maternal plasma. These shortlisted markers are potentially useful for monitoring pregnancy noninvasively. Since the functions of most of the miRNAs remain largely unexplored, I have applied this systematic search which required no prior knowledge of the miRNA function.

Among the five placental miRNAs being studied in Chapter 5.3.2, each of them had a different detection rates, concentrations and clearance kinetics upon delivery from

one another. For example, in one of the placental miRNAs, miR-141, it was shown to be readily detectable in all three trimesters of pregnancy with the earliest detection in the 12^{th} week of gestation. Its concentration is reasonably high and increases with gestational age. Moreover, its concentration decreases significantly (Wilcoxon test, $P = 0.003$) with a median fold change of 5.3. However, it was noted that miR-141 did not show complete clearance from all pregnant women after delivery. A possible explanation for this is that miR-141 is being cleared from maternal plasma at different rates from different women. Nevertheless, such a robust detection of the miR-141 in maternal plasma made it potentially useful for the noninvasive monitoring of pregnancy. Similar investigation on the biology of other placental miRNA should be carried out to address their potentials.

In subsequent parts of this study, I sought to characterize the various molecular characteristics of circulating placental miRNAs. MiR-141 was chosen as a target for the study because it has a higher plasma concentration than the other candidates on the list.

Given the labile nature of RNA and the ribonuclease activity in the blood (Reddi and Holland 1976), exogenous and purified mRNA added to the plasma was degraded within seconds (Tsui *et al.* 2002). Surprisingly, circulating mRNA exists in detectable quantities in plasma. Tsui *et al.* (2004) demonstrated that such an exceptional stability was a result of the association between the mRNAs and subcellular particles. Because miRNA, like mRNA, was shown to be stably present in plasma and exists at an exceptionally high level, it raises the question of whether the stability of circulating miRNAs may also result from an association with subcellular particles. To address this problem, I performed a filtration study. The data clearly indicated that

filtration had no significant effect on the concentration of miR-141 in maternal plasma. These results demonstrated that miR-141 in plasma is not associated with subcellular particles with size larger than 0.22 μm. In contrast, the *hPL* mRNA, which was studied in parallel as a control, demonstrated its association with subcellular-particles as described previously. In brief, the above results suggest that the exceptional stability of circulating miRNA is not a result of the association with subcellular particles. However, whether this exceptional stability of miRNAs is a result of association with particulate matter, such as protein factors and RNA-induced silencing complex, remains to be elucidated, perhaps by the use of ultra-centrifugation studies (Tsui *et al.* 2002). Moreover, an answer as to whether this special stability of miRNA is a general phenomenon awaits similar studies using other placental miRNA species.

To further compare the stability of mRNA and miRNA in plasma, I performed a stability test. Extracted placental RNA was exogenously added and incubated in the plasma of a healthy male. Because extracted RNA is not associated with any subcellular or particulate matter, it is reasoned that the experiment will give a fair comparison of the stability of the two species of RNA as attributed to the length of the different species. Since there were no detectable concentrations of *hPL* mRNA and miR-141 in the male plasma, any *hPL* mRNA and miR-141 detected in this plasma sample were derived from the exogenously added placental RNA preparation. The data indicate that exogenous, purified miRNA added to the plasma was more resistant to degradation and remained at substantial concentration even after two hours of incubation, especially when compared with *hPL* mRNA. The explanation for these results is not clear at present. One possible explanation is that miRNAs may be too short to be further cleaved by ribonucleases in plasma, thus allowing them to

exist in particle-free form in plasma. Nonetheless, the exceptional stability of miRNA in plasma is a definite advantage for its potential application in noninvasive prenatal monitoring.

In conclusion, the present study has demonstrated miRNAs as a potential novel class of gender- and polymorphism-independent pregnancy-associated markers in maternal plasma. They are not associated with subcellular-particle. Yet, they exist stably in plasma and could be detected quantitatively and robustly even after freezing, thawing and processing of clinical blood samples routinely. I have validated a method for the discovery of such miRNAs, and a list of such miRNAs has also been complied. With the help of higher throughput technologies, such as sensitive labeling and detection strategies for miRNA microarray (Wang *et al.* 2007), this search strategy will allow discovery of more placental miRNA in maternal plasma with diagnostic potential. They would facilitate the development of plasma miRNA as diagnostic markers. For example, with the recent demonstration of upregulated expression of miR-210 and miR-182 in preeclamptic placentas over gestational age matched normal placenta (Pineles *et al.* 2007), it would be interesting to investigate if the plasma concentration of these miRNAs would also be aberrantly represented. In addition, the demonstration of cell-free placental miRNAs in maternal plasma would initiate further investigations on its biological significance. Besides, as miRNAs derived from a tumor might analogously be released into the plasma, I believe that this study has opened up possibilities in the development of cell-free miRNAs in plasma for tumor diagnosis.

SECTION IV: DETECTION OF CELL-FREE DNA IN URINE

CHAPTER 6: HEMATOPOIETIC STEM CELL TRANSPLANTATION RECIPIENTS AS A MODEL TO STUDY CELL-FREE DNA IN URINE

6.1 Introduction

In recent years, the biology and diagnostic applications of cell-free DNA in plasma have attracted much research interest (Lo and Chiu 2007). In particular, Lo *et al.* (1999e) demonstrated the kinetics of rapid clearance of circulating fetal DNA in plasma after delivery of the fetuses. A number of hypotheses were raised to address the mechanism behind this phenomenon. One hypothesis was made by Botezatu *et al.* (2000). The group proposed that a portion of these cell-free DNA was cleared from the plasma as it passed through the kidney barrier into the urine. This group provided a number of evidences for this phenomenon. Firstly, in the urine of mice injected with human DNA, the *Alu* sequences, which is present only in the human genome, was successfully detected (Botezatu *et al.* 2000). In addition, in women pregnant with male fetuses, male-specific sequences could be detected (Botezatu *et al.* 2000). They also detected, in the urine of cancer patients with colon adenocarcinoma, the cancer-associated *K-ras* mutations DNA sequences (Botezatu *et al.* 2000). Since then, the term 'transrenal-DNA' (Tr-DNA) has been coined to describe such DNA species which have been passed from the plasma into the urine through the kidney barrier.

Transrenal DNA in urine is different from cell-free DNA in the urine which is derived from tumor of the urinary tract. In principle, the passage of these cell-free DNA molecules derived from tumors of the urinary tract does not require the transrenal passage of cell-free DNA. Of particular significance, Su *et al.* (2004b)

demonstrated that unfractionated human urine consists of both high-molecular weight DNA and low-molecular weight DNA. Upon centrifugation to remove cell debris, the concentration of low-molecular weight DNA was enriched in the supernatant fraction, while that of the high-molecular weight DNA was dramatically decreased (Su *et al.* 2004b). Also, in about 83% of colorectal cancer patients, K-*ras* mutations concordant with those found in the primary tumours could be detected in the urine, and such K-*ras* mutation sequences were abundant in the low-molecular weight DNA, but far less abundant in the high-molecular weight DNA isolated from the urine (Su *et al.* 2004b). Taken together, these data suggested that cell-free DNA species in the urine are short in size and were estimated to be about 150 to 250 bp in length (Su *et al.* 2004b).

Independent studies were published reporting successful detections of male-specific DNA sequences in the urine of a proportion of pregnant women with male fetuses (Al-Yatama *et al.* 2001, Koide *et al.* 2005), and hence lending further support to the transrenal DNA hypothesis. However, some investigator reported the inability to detect male-specific DNA sequences in women pregnant with male fetuses (Zhong *et al.* 2001a). In particular, the same group was also unable to detect these male-specific DNA sequences in pregnant women, who were suffering from pre-eclampsia, with male fetuses (Li *et al.* 2003). It was previously shown that in pregnant women with pre-eclampsia, elevated levels of fetal DNA in their plasma (Zhong *et al.* 2000) and increased renal permeability were observed. And proteinuria was one of the complications involved (Hayashi *et al.* 2002). Therefore, these reports concerning the inability to detect fetal DNA in urine of pregnant women have raised considerable doubts concerning the feasibility of Tr-DNA analysis.

In fact, several technical challenges have to be overcome to facilitate the successful detection of cell-free DNA populations, including Tr-DNA, in urine. Firstly, it has been reported that nucleases in urine reduces the efficiency of DNA analysis (Milde *et al.* 1999). Thus, ethylenediaminetetraacetic acid (EDTA) was added to reduce the activities of these divalent ion-dependent DNA nucleases. Secondly, cell-free DNA populations in the urine have been reported to contain a fraction of DNA species that are relatively short in size, roughly 150 to 250 bp (Su *et al.* 2004b). Thus, investigators have proposed the use of special DNA extraction methods to retain these short DNA fragments (Su *et al.* 2004a), which are often washed away as "impurities" in the most popular silica column-based DNA extraction kits. Moreover, PCR of short amplicon sizes are more preferred to long ones for DNA detection (Su *et al.* 2004a). Given all these technical considerations, there is currently no consensus method for processing and extracting DNA from urine. Subsequently, these factors have been hindering the understanding of cell-free DNA in urine.

To gain more insights into the constituting components of cell-free DNA species, including Tr-DNA, in the urine, I analyzed them with molecular methods using the sex-mismatched hematopoietic stem cell transplantation (HSCT) recipients as a study model. Previous data have shown that following sex-mismatched HSCT, the 'gender' of the recipient's plasma DNA changed and resembled that of the donor (Lui *et al.* 2002). For example, in female recipients of male stem cells, the median fractional concentration of donor-derived male DNA was 59.5% (interquartile range 42 to 65%), while her urinary tract would remain "female". This fractional concentration of donor-derived DNA in HSCT recipients was much higher than the mean fractional concentration of fetal DNA in maternal plasma, which was only 3.4% during weeks 11 to 17 of gestation and 6.2% in the third trimester of pregnancy (Lo *et al.* 1997),

and less variable than that in plasma of cancer patients (Anker *et al.* 1997). Thus, these results suggested that sex-mismatched HSCT may be a more powerful model system for the investigation on Tr-DNA because, as postulated by the phenomenon of Tr-DNA, donor DNA would be more readily detected in the urine of the HSCT recipients than in other transrenal models. It is hoped that this HSCT model can further our understanding of the molecular characteristics of cell-free urine DNA, including Tr-DNA, and hence facilitate the future development of cell-free urine DNA for noninvasive diagnosis.

With a newly devised MassEXTEND™ assay accurately quantifying the fractional concentration of donor-derived DNA and a set of QPCR assays studying DNA size distribution, I dissected and observed that there are at least two sub-populations of cell-free DNA species in the urine.

6.2 Materials and methods

6.2.1 Sample collection

Peripheral blood and urine samples were collected from 11 patients who suffered from hematological cancer or other hematological diseases and who received HSCT from donors of the opposite gender (Table 6.1). All the samples were prepared using the protocol described in Chapter 3.1. All patients were recruited with informed consent for the Department of Paediatrics, Prince of Wales Hospital, Shatin, Hong Kong. They were all in clinical remission from their respective diseases. The study was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee.

6.2.2 Experimental design

Validation of the zinc finger protein gene assay for determining the fractional concentrations of male DNA in a mixture of male and female DNA

The *zinc finger protein gene* assay was designed as described in Chapter 3.4.2 to determine the fractional concentration of male DNA in mixtures of male and female DNA. To evaluate the precision of this assay, I replicated the quantification of fractional concentration of male DNA in urinary DNA from a healthy male subject for 20 times as described in Chapter 3.4.2. The analytical intrassay coefficient of variation percentage (CV%) of the fractional concentrations obtained was used as reference of analysis.

To evaluate the quantitative linearity of the assay, a series of artificial DNA mixtures with different and known fractional concentrations of male DNA and female DNA were prepared. Fractional concentrations in different mixtures were measured using the assay as described in Chapter 3.4.2. The regression correlation coefficient (R^2) of the measured and expected fractional concentrations in the mixtures was used as the reference of analysis.

Fractional concentration of male DNA in peripheral blood cells, cell-free plasma, cell-free urine and urine cell pellets of sex-mismatched HSCT patients

In order to decipher the constituting DNA components in urine using the sex-mismatched HSCT model, plasma, blood cells, urine and urine cell pellets from 11 sex-mismatched HSCT patients were measured for the fractional concentrations of male DNA using the *zinc finger protein gene* assay as described in Chapter 3.4.2. I then performed statistical tests to show, if there was any relationship between the

constituting components of different compartments.

Size distribution of cell-free DNA in the plasma and the urine of sex-mismatched HSCT patients

In order to analyze the size distribution of cell-free DNA molecules in the plasma and the urine, these bodily fluids were collected from 11 sex-mismatched HSCT patients. To measure the size distribution of the total population of cell-free DNA in these bodily fluids, the concentrations of the *SRY* (with amplicon sizes of 63, 107 and 377 bp) and *LEP* (with amplicon sizes of 63, 105 and 356 bp) genes using QPCR assay as described in Chapter 3.3.4.2. This gave information of the cell-free DNA in urine in terms of the fragmentation of the cell-free DNA. The relative concentrations of *SRY* and *LEP* gene in each sample were used for analysis of the size distribution of the cell-free DNA. The relative concentrations were calculated by dividing the absolute concentration of DNA determined by a real-time QPCR system for a particular amplicon size by the absolute concentration of DNA determined in the PCR system with the shortest amplicon used (Chan *et al.* 2004). In this study, the shortest amplicon used was 63 bp for both of the *LEP* and *SRY* genes.

6.2.3 DNA extraction and quantification

Processing of peripheral blood and urine samples for DNA followed the procedure as described in Chapter 3.1. Quantification of fractional concentration of male DNA followed the procedure as described in Chapter 3.4.2. Quantification of cell-free DNA of *SRY* and *LEP* genes of different amplicons size followed the procedure as described in Chapter 3.3.4.2. Serial dilutions of male blood cells genomic DNA with concentrations ranging between 0.78 and 10,000 genome equivalents per five microliters were used for construction of standard curve of absolute quantification of

the DNA.

Table 6.1. Cellular chimerism in sex-mismatched bone marrow transplantation patients.

Patient	Gender of donor	Gender of recipient	Diagnosis	Months after transplantation	Percentage of male DNA in blood cells (%)[b]	Percentage of donor-derived cells in blood (%)[c]
B003	F	M	T-cell ALL[a]	16	0.96	99.7
B008	F	M	Beta thalassemia major	151	0.00	99.7
B009	F	M	Chronic granulomatous disease	168	0.15	99.5
B016	F	M	Pre-B cell ALL	36	0.00	99.5
B025	F	M	AML	9	0.00	99.5
B026	F	M	Wiskcott/Aldrich Syndrome	42	0.00	99.3
B028	F	M	Adrenoleukodystrophy	3	0.19	99.5
B001	M	F	Philadelphia Chr +ve ALL	60	90.29	99.5
B007	M	F	Severe Aplastic Anemia	74	93.40	99.7
B013	M	F	Beta thalassemia major	24	96.36	99.3
B015	M	F	Philadelphia Chr +ve ALL	135	98.90	99.4

[a]ALL; acute lymphoblastic leukemia
[b]Percentage was given by *zinc finger protein gene* assay
[c]Percentage was given by fluorescence *in situ* hybridization analysis

6.3 Results

6.3.1 Validation of the *zinc finger protein gene* assay

In this study, the *zinc finger protein gene* assay was used to determine the fractional concentration of male DNA in mixtures of male and female DNA. This assay involves the use of a single PCR system to amplify the two homologues of the *zinc finger protein gene* located on the X- and Y-chromosomes, and generates two similar PCR products distinguishable by MALDI-TOF MS (Chapter 3.4.2). Compared to other analytical systems, in which one PCR system amplifies the male DNA and another one amplifies the female DNA, this analytical system maintains nearly identical PCR efficiencies in amplifying the X- and Y-chromosomes for estimating the male to female DNA ratio in term of fractional concentration of male DNA. Thus, the estimation is more accurate. Using this assay, the expected fractional concentration of a male DNA sample is 100% while that for a female DNA sample is 0%.

To assess the precision of the *zinc finger protein gene* assay, 20 replicated quantifications of the fractional concentration of male DNA in urine cell-free DNA from a healthy male subject were performed. The mean measured fractional concentration was 92% (mean ± SD, 92% ± 4%). The CV% for the fractional concentrations of these replicates was 4.9%. The assay showed a reasonably high precision in detection.

To assess the quantitative linearity of this assay, a series of artificial DNA mixtures with known fractional concentrations of male DNA were analyzed using the *zinc finger protein gene* assay. When the expected and measured fractional concentrations

of male DNA were plotted against each other, the assay had an R^2 of 0.99 and a slope of 0.96 (Figure 6.1). The assay was highly quantitative with a detection range of fractional concentration of male DNA between 0 and 100%.

6.3.2 Fractional concentration of male DNA in blood cells and plasma of sex-mismatched HSCT patients

Because this study involves the detection of donor-derived DNA in plasma and urine, I first demonstrated that the blood cells of all the HSCT recipients under study were completely donor-derived. This would facilitate the comparison of data between different subjects. As determined by the *zinc finger protein* gene assay, fractional concentration of male DNA in the blood cells of recipients showed that the "gender" of the blood cells of the recipients shifted completely to that of the donors. For example, in female recipients of male stem cells, the blood cells were shown to contain about 100% of male DNA and thus were mainly composed of male DNA; in male recipients of female stem cells, the blood cells were shown to contain about 0% of male DNA and thus were mainly composed of female DNA. This is consistent with the FISH results which showed that all blood cells in all the recipients were nearly 100% donor-derived (Table 6.1). Taken together, they showed that blood cells of all the HSCT recipients under this study were essentially completely donor-derived.

Using the *zinc finger protein* gene assay, I illustrated the chimerism status of donor- and recipient-derived cell-free DNA in the plasma of sex-mismatched HSCT recipients. It was previously shown that in HSCT recipients, majority of the cell-free DNA in plasma was donor-derived (Lui *et al.* 2002) and a minority of the cell-fee DNA was recipient-derived (Lui *et al.* 2003). Thus, this part of the study aimed at

producing similar results to establish the feasiblility of using of this newly devised assay in DNA chimerism analysis.

It was found that the median fractional concentration of male DNA in the plasma of the group of female recipients of male stem cells was 79.3 % (Figure 6.2A). In this group, the 79.3% of male DNA detected could only originate from the male blood cells. Therefore, this 79.3% represented the fraction of cell-free DNA originating from the hematopoietic system. The difference between the 79.3% in plasma and the 100% as in donor-derived blood cells indicated that a portion of plasma DNA originated from the female nonhematopoietic cells of the recipients.

In contrast, the median fractional concentration of male DNA in the plasma of the group of male recipients of female stem cells was 27.2% (Figure 6.2A). In this group, the 27.2% of male DNA of the cell-free DNA in the plasma represented DNA originating from the male nonhematopoietic cells and/or the remaining male hematopoietic cells of the male recipients. Because in all the patients under this study, essentially all of the blood cells were donor-derived, I could deduce that this 27.2% represented the percentage of cell-free DNA in the plasma originating from the male nonhematopoietic cells throughout the body.

Taken together, these results were consistent with the previously published data which suggested that hematopoietic cells are the predominant source of cell-free DNA in plasma (Lui *et al.* 2002) while nonhematopoietic cells accounted for a minority of the cell-free DNA in plasma (Lui *et al.* 2003). These results also confirmed that the *zinc finger protein* gene assay could be used for study DNA chimerism.

6.3.3 Fractional concentration of male DNA in the urine and the urine cell pellets of sex-mismatched HSCT patients

I further sought to decipher the constituting components of cell-free DNA in the urine as derived from the donor and the recipient using the *zinc finger protein gene* assay. As shown in Figure 6.2B, the median fractional concentration of the male DNA in the urine of the group of female recipients of male stem cells was 38.3% while that for the group of male recipients of female stem cells was 92.3%. When comparing the median fractional concentrations of male DNA in the plasma and the urine, it was found that the fractional concentration of the recipient-derived DNA increased as the plasma passed through the kidney barrier into the urine. For example, in female recipients of male stem cells, there were 79.3% and 38.3% of male DNA in the plasma and the urine, respectively, representing a 2-fold increase in the fractional concentration of recipient-derived DNA in the urine than in the plasma; in male recipients of female stem cells, there were 27.2% and 92.3% of male DNA in the plasma and the urine, respectively. In other words, 72.8% and 7.7% of female DNA were detected in the plasma and the urine, respectively, and it represented a 9-fold increase in the fractional concentration of recipient-derived DNA in the urine than in the plasma.

According to the hypothesis of Tr-DNA formation, Tr-DNA in urine is derived from cell-free DNA in plasma after it passes through the kidney barrier. I performed correlation tests to see if there was correlation between the fractional concentrations of male DNA in the urine and those in the plasma. If cell-free DNA in the urine is mainly Tr-DNA derived from cell-free DNA in the plasma, a significant correlation between the two groups would be expected. Table 6.2 summarizes the fractional

concentrations of male DNA in the plasma, urine and urine cell pellet. There was no significant correlation between the fractional concentrations of male DNA in the plasma and those in the urine (Spearman rank order correlation, r_s = -0.427, p = 0.178 > 0.05, Figure 6.3A). However, the fractional concentrations of male DNA in the urine and those in the urinary cell pellets showed a statistically significant correlation (Spearman rank order correlation, r_s = 0.682, p = 0.0186 < 0.05, Figure 6.3B). This data suggested the possible contribution of urine cell pellets to the urine cell-free DNA. Notably, it also provided a possible explanation for the much larger increase in the proportion of recipient-derived DNA in urine as cell-free DNA entered urine from plasma, in male recipients of female stem cells (9-fold increase) as compared with female recipients of male (2-fold increase) as described in the previous section. That is, the much longer urinary tract in male than in female might contribute to more recipient cells in the cellular portion of the urine.

6.3.4 Size distribution of cell-free DNA in peripheral blood and urine samples of sex-mismatched HSCT patients

To further decipher the constitution of cell-free DNA in the urine, I performed a series of real-time quantitative PCR assays to study the size distribution of cell-free DNA molecules in the urine. It was previously shown that majority of cell-free DNA molecules in plasma has less than 201 bp (Chan *et al.* 2004). Therefore, if cell-free DNA in the urine is derived from cell-free DNA in the plasma, it should also mainly consist of short DNA molecules. If there is a substantial amount of cell-free DNA molecules in urine which is longer than 300 bp, they are probably released from the cellular portion of the urine.

In this thesis, the real-time quantitative PCR assays were able to detect down to one

genome equivalent (GE) of the *LEP* gene or the *SRY* gene for all the amplicon sizes (Chan *et al.* 2004). Because the absolute DNA concentrations vary widely between different individuals, I calculated the relative concentrations (Chapter 6.2.2) for each amplicon size so that the size distribution of DNA in each individual could be compared and summarized.

For the cases with male donors to female recipients, the size distribution of cell-free DNA in the plasma and the urine were summarized in Table 6.3A. In the plasma, I observed that when the PCR amplicon size for *LEP* increased from 63 bp to 105 bp to 356 bp, its median concentrations, relative to the 63-bp amplicon, decreased from 100% to 50.7% to 22.3% (median absolute concentration of *LEP* with the 63 bp assay was 184.7 GE/mL). This implied that the total, both donor- and recipient-derived, DNA molecules in cell-free plasma were mainly short DNA fragments. These data were consistent with the previous findings which showed that the majority (86%) of cell-free DNA in the plasma was smaller than 201 bp (Chan *et al.* 2004). In the urine, I observed that when the PCR amplicon size for *LEP* increased from 63 bp to 105 bp to 356 bp, its median concentrations, relative to the 63-bp amplicon, similarly decreased from 100% to 93.0% to 43.9% (median absolute concentration of *LEP* with the 63 bp assay was 1775.6 GE/mL). Thus, the total, both donor- and recipient-derived, DNA molecules in cell-free urine were also mainly short DNA fragments. In particular, I observed that in both of the 105 bp and 356 bp *LEP* assays, the relative concentrations of all cases were higher in the urine (median, 93.9% for the 105-bp amplicon and 43.9% for the 356-bp amplicon) than in the plasma (median, 50.7% for the 105-bp amplicon and 22.3% for the 356-bp amplicon). It showed that *LEP* DNA molecules, which represented both of the donor- and recipient-derived DNA, were relatively longer in the urine than in the plasma.

Moreover, because it has become previously thought that the size of Tr-DNA is mainly between 150 bp and 250 bp (Su *et al.* 2004b), the existence of more 356 bp *LEP* DNA molecule in the urine than in the plasma suggested a release of these high-moleculer weight DNA into the urine from the cellular portion of the urine.

In the plasma, I observed that when the PCR amplicon size for *SRY* increased from 63 bp to 107 bp to 377 bp, its median concentrations, relative to the 63-bp amplicon, decreased from 100% to 55.4% to 0% (median absolute concentration of *SRY* with the 63 bp assay was 116.3 GE/mL). This implied that the donor-derived DNA molecules in cell-free plasma were mainly short DNA fragments. In the urine, I observed that when the PCR amplicon size for *SRY* increased from 63 bp to 107 bp to 377 bp, its median concentrations, relative to the 63-bp amplicon, similarly decreased from 100% to 83.8% to 15.6% (median absolute concentration of *SRY* with the 63 bp assay was 376.1 GE/mL). Thus, the donor-derived DNA molecules in the cell-free urine were also mainly short DNA fragments. In particular, I observed that in both of the 107 bp and 377 bp *SRY* assays, the relative concentrations of all except one cases were higher in the urine (median, 83.8% for the 107-bp SRY amplicon and 15.6% for the 377-bp amplicon) than in the plasma (median, 55.4% for the 107-bp SRY amplicon and 0.0% for the 377-bp amplicon). It showed that *SRY*, which represented donor-derived DNA from blood cells, were relatively longer in the urine than in the plasma. Moreover, the existence of *SRY* DNA molecule longer than 377 bp in the urine but not in the plasma suggested a direct release of these high-moleculer weight DNA molecules from donor-dervied blood cells in the urine.

For cases with female donors to male recipients, the size distribution of cell-free DNA in the plasma and the urine were summarized in Table 6.3B. In the plasma, I

observed that when the PCR amplicon size for *LEP* increased from 63 bp to 105 bp to 356 bp, its median concentrations, relative to the 63-bp amplicon, decreased from 100% to 96.9% to 0% (median absolute concentration of *LEP* with the 63 bp assay was 594.1 GE/mL). This implied that the total, both donor- and recipient-derived, DNA molecules in cell-free plasma were mainly short DNA fragments. In the urine, I observed that when the PCR amplicon size for *LEP* increased from 63 bp to 105 bp to 356 bp, its median concentrations, relative to the 63-bp amplicon, similarly decreased from 100% to 83.3% to 1.8% (median absolute concentration of *LEP* with the 63 bp assay was 632.7 GE/mL). Thus, the total, both donor- and recipient-derived, DNA molecules in cell-free urine were also mainly short DNA fragments. In summary, the existence of more 356 bp *LEP* DNA molecules in the urine than in the plasma suggested a release of these high moleculer weight DNA into the urine from cellular portion of the urine. However, unlike the cases with female recipients receiving male stem cells, the *LEP* DNA molecular with 105 bp amplicon showed a higher median relative concentration in the plasma than in the urine (96.9% and 83.3%, respectively). It might be due to increased level of apoptosis, which release small cell-free DNA fragments into plasma, in the donor-derived blood cells or solid organ of the recipients after HSCT. However, because the exact sources of these short *LEP* DNA molecules cannot be determined, it awaits further investigation to clearly address the sources of such increased release of *LEP* DNA.

In the plasma, I observed that when the PCR amplicon size for *SRY* increased from 63 bp to 107 bp to 377 bp, its median concentrations, relative to the 63-bp amplicon, decreased from 100% to 54.5% to 0% (median absolute concentration of *SRY* with the 63 bp assay was 103.4 GE/mL). This implied that the recipient-derived DNA molecules in cell-free plasma were mainly short DNA fragments. In the urine, I

observed that when the PCR amplicon size for *SRY* increased from 63 bp to 107 bp to 377 bp, its median concentrations, relative to the 63-bp amplicon, similarly decreased from 100% to 65.8% to 8.9% (median absolute concentration of *SRY* with the 63 bp assay was 647.12 GE/mL). Thus, the recipient-derived DNA molecules in cell-free urine were also mainly short DNA fragments. In particular, I observed that in both of the 107 bp and 377 bp *SRY* assays, the median relative concentrations were higher in the urine than that in the plasma. It showed that *SRY*, which represented recipient-derived DNA, were relatively longer in the urine than in the plasma. Moreover, the existence of *SRY* DNA molecule longer than 377 bp in the urine but not in the plasma suggested a direct release of these high-moleculer weight DNA from cellular portion of the urine.

In summary, it could be observed that, in general, the sizes of cell-free DNA molecules, both *LEP* and *SRY*, in the urine were longer than those in the plasma. Of particular significance, the existence of *SRY* DNA molecules longer than 377 bp in urine, but not in plasma, of female recipients of male stem cells, suggested the direct release of these high-molecular weight DNA molecules into urine from donor-derived blood cells liberated into urine from the circulation. Similarly, the existence of *SRY* DNA molecules longer than 377 bp in urine, but not in plasma, of male recipients of female stem cells, suggested the direct release of these high-molecular weight DNA molecules into urine from recipient-derived cells liberated into urine from the urinary tract.

Table 6.2 Fractional concentrations of male DNA (%) in the plasma, urine and urine cell pellet of sex-mismatched HSCT recipients.

Sample	Fractional concentration of male DNA (%)		
	Plasma	Urine	Urine cell pellet
Male donor, female recipient			
B001	64.9	36.5	42.5
B007	90.7	40.1	15.5
B013	103.6	26.0	42.2
B015	68.0	88.1	40.8
Median	***79.3***	***38.3***	***41.5***
Female donor, male recipient			
B003	30.4	78.1	40.7
B008	27.2	70.8	41.6
B009	0.0	92.3	83.7
B016	23.0	88.8	90.6
B025	71.3	108.0	101.0
B026	35.2	111.1	84.5
B028	26.0	102.3	94.6
Median	***27.2***	***92.3***	***84.5***

Table 6.3 Relative concentrations (%) of *SRY* and *LEP* DNA in the plasma and urine of sex-mismatched HSCT recipients.

(A) Male donors, female recipients

	SRY			*LEP*		
Amplicon size	**63 bp**	**107 bp**	**377 bp**	**63 bp**	**105 bp**	**356 bp**
Plasma						
B001	100.0	30.0	0.0	100.0	68.9	0.0
B007	100.0	64.1	0.0	100.0	63.1	0.0
B013	100.0	46.6	0.0	100.0	36.0	64.2
B015	100.0	109.3	9.2	100.0	38.3	44.5
Median	***100.0***	***55.4***	***0.0***	***100.0***	***50.7***	***22.3***
Urine						
B001	100.0	69.1	15.8	100.0	93.2	4.8
B007	100.0	91.6	13.7	100.0	93.2	1.5
B013	100.0	84.3	15.6	100.0	94.5	83.0
B015	100.0	83.4	15.6	100.0	95.9	100.4
Median	***100.0***	***83.8***	***15.6***	***100.0***	***93.9***	***43.9***

(B) Female donors, male recipients

	SRY			*LEP*		
Amplicon size	**63 bp**	**107 bp**	**377 bp**	**63 bp**	**105 bp**	**356 bp**
Plasma						
B003	100.0	88.7	0.0	100.0	70.2	0.0
B008	100.0	0.0	0.0	100.0	96.9	0.0
B009	100.0	0.0	0.0	100.0	81.4	0.0
B016	100.0	54.5	0.0	100.0	107.1	0.0
B025	100.0	55.0	0.0	100.0	97.9	0.0
B026	100.0	49.9	0.0	100.0	118.1	1.2
B028	100.0	123.9	0.0	100.0	89.8	1.6
Median	***100.0***	***54.5***	***0.0***	***100.0***	***96.9***	***0.0***
Urine						
B003	100.0	45.3	4.3	100.0	83.3	5.7
B008	100.0	80.4	19.3	100.0	85.3	4.9
B009	100.0	73.2	15.7	100.0	103.9	1.8
B016	100.0	44.4	2.7	100.0	80.7	0.3
B025	100.0	65.8	8.9	100.0	93.9	0.0
B026	100.0	60.0	4.7	100.0	80.5	0.9
B028	100.0	72.8	13.8	100.0	82.3	19.1
Median	***100.0***	***65.8***	***8.9***	***100.0***	***83.3***	***1.8***

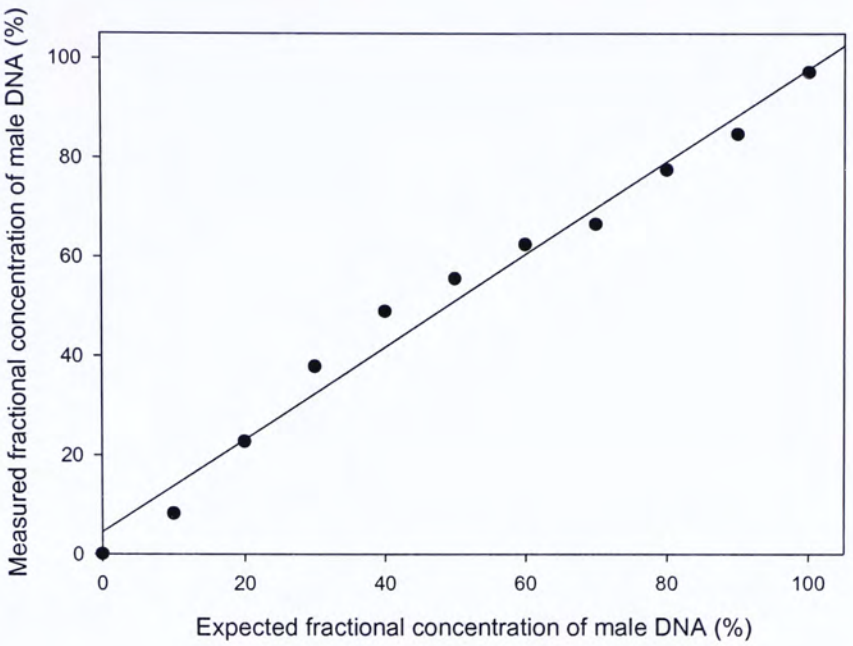
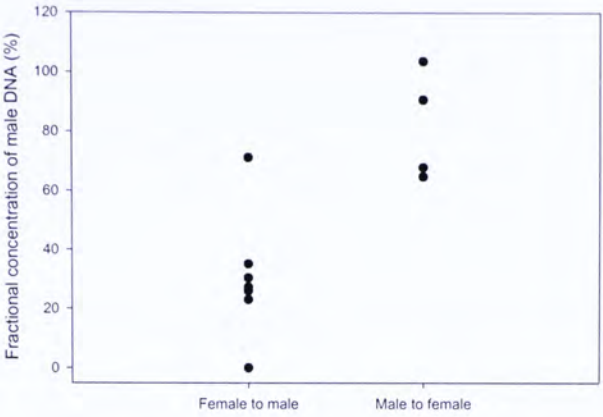


Figure 6.1 Standard curve of *zinc finger protein gene* assay on a series of artificial mixtures of male and female DNA.

Plot of the measured fractional concentrations of male DNA (%) against expected fractional concentrations of male DNA (%). The correlation coefficient is 0.99.

(A)



(B)

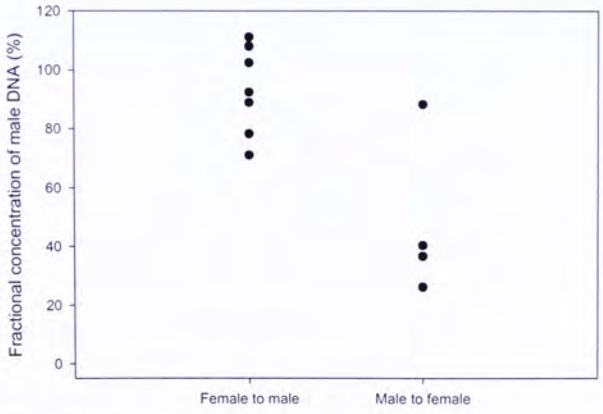
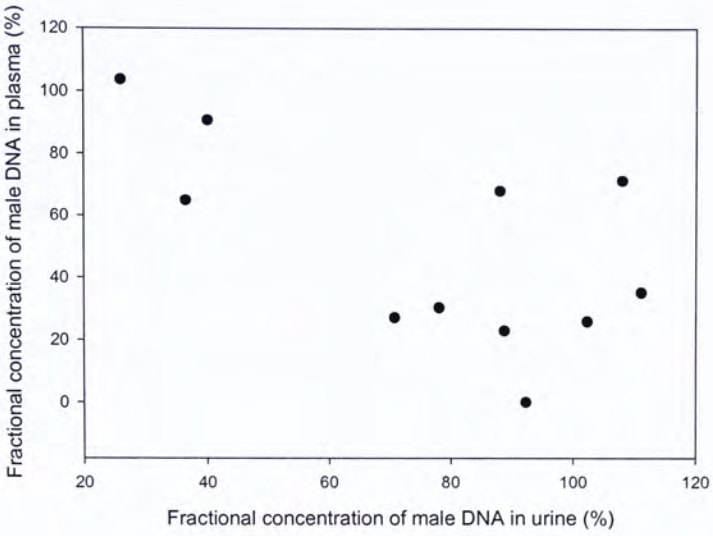


Figure 6.2 Fractional concentrations of male DNA (%) in plasma and urine.

Dot plots of fractional concentrations of male DNA in the plasma ***(A)*** and urine ***(B)*** of sex-mismatched HSCT patients. The genders of donors and recipients are shown on the *x axis*. The fractional concentration of the male DNA in the plasma is plotted on the *y axis*. The percentage of male DNA was generated using the *zinc finger protein gene* assay.

(A)



(B)

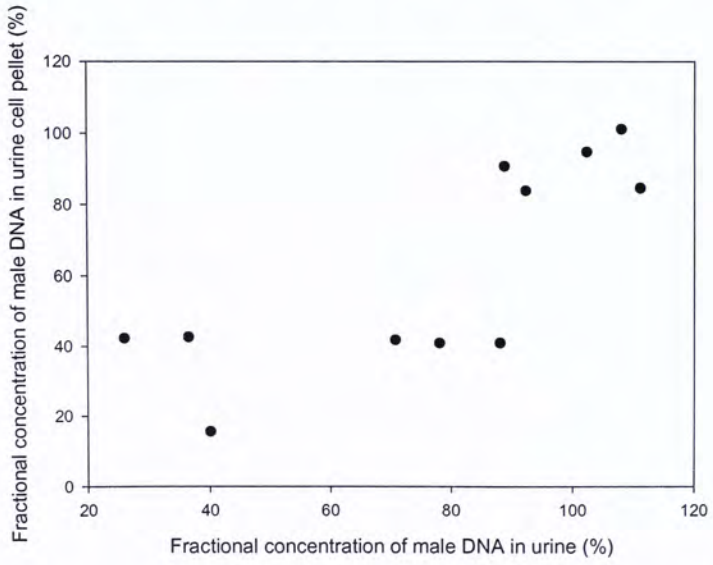


Figure 6.3 Fractional concentrations of male DNA (%) in sex-mismatched HSCT patients.

(A) Correlation between fractional concentration of male DNA (%) in plasma and urine. ***(B)*** Correlation between fractional concentration of male DNA (%) in urine cell pellet and urine.

6.4 Discussion

In this part of the thesis, I studied the different constituting components of cell-free DNA in the urine of HSCT recipients in the hope of providing technical and biological information on the use of these cell-free DNA, especially the Tr-DNA, in urine for noninvasive molecular tests.

According to the Tr-DNA hypothesis (Botezatu *et al.* 2000), Tr-DNA is downstream from cell-free DNA in plasma as it passes through the kidney barrier. Thus, only the more finely fragmented cell-free DNA in plasma can appear as Tr-DNA. In pregnant women, only a minor proportion (3.4%-6.2%) (Lo *et al.* 1998b) of fetal cell-free DNA appears in maternal plasma. As fetal DNA passes the kidney barrier, its concentration further decreases as larger fragments were filtered. It might be the reason of the inability of some investigators to detect fetal DNA in urine of pregnant women (Zhong *et al.* 2001a, Li *et al.* 2003). In cancer patients, although the proportion of tumor-derived DNA in plasma is reasonably high, such chimerism varies a lot between different patients (Anker *et al.* 1997). Thus, they might not be ideal models for studying the phenomenon of Tr-DNA. To address this problem, this study employed the sex-mismatched HSCT model which has higher levels and smaller range of fractional concentration of donor-derived DNA in plasma than fetal DNA is in maternal plasma (Lui *et al.* 2002). Thus, more donor-derived DNA is expected to pass into urine, which in turn, allows more robust detection. The implementation of this HSCT model has the potential to greatly improve current understandings on the phenomenon of Tr-DNA and the different cell-free components in the urine.

To gain knowledge on the different constituting cell-free DNA components in urine, I

studied the fractional concentration of cell-free male DNA in the plasma, urine and urine cell pellets of these sex-mismached HSCT recipients. Size distributions of DNA molecules were also studied. It was found that a number of possible pathways may contribute to the cell-free DNA in urine (Figure 6.4). Firstly, it is the transrenal passage of cell-free DNA from the plasma into the urine, i.e. Tr-DNA. In female recipients of male stem cells, male DNA was present in both of the plasma and the urine of the recipients (Figure 6.2). Thus, it provided evidences to support for the presence of Tr-DNA. Secondly, it is the release of high-molecular weight DNA from blood cells liberated into the urine from the circulation. In female recipients of male stem cells, the existence of donor-derived *SRY* DNA molecules longer than 377 bp in urine but not in the plasma (Table 6.3A) suggested such a release of these high-molecular weight cell-free DNA from blood cells into the urine. Thirdly, it is the release of high-molecular weight DNA from cells liberated into the urine from the urinary tract, e.g. epithelial cells. Because in female recipients of male stem cells, the identity of DNA molecules released from the female urinary tract cannot be distinguished from that of the male donor. The third pathway was illustrated in male recipients of female stem cells, whose DNA from urinary tract is "male". In Table 6.3B, the existence of recipient-derived *SRY* DNA molecules longer than 377 bp in urine but not in plasma suggested such a release of high-molecular weight cell-free DNA from cells liberated into the urine from the urinary tract.

Summarizing the three mechanisms mentioned above, the transrenal passage of plasma cell-free DNA, cells from the hematopoietic system or the urinary tract are possible contributors of cell-free DNA in urine. To determine which mechanism is more prevalent in general, I performed correlation tests of the fractional concentrations of male DNA in the urine, urine cell pellets and plasma in these

sex-mismatched HSCT recipients. In these tests, a significant correlation is observed between the fractional concentrations in the cell-free urine and that in the urine cell pellets, but not between the fractional concentrations in the cell-free urine and the cell-free plasma. Therefore, I could deduce a dominant contribution of the cell-free DNA in urine from the cellular portion of urine.

In summary, in this part of the thesis, I have used the HSCT model to study the constituting components of cell-free DNA in urine. It was found that the majority of cell-free DNA in urine was derived from cellular portion of the urine, which consisted of both donor- and recipient-derived cells. Moreover, it was shown that cell-free DNA in urine originated from hematopoietic cells in the circulation could be easily detected in urine using a PCR-based technology. Thus, this study provided fundamental information for the use of cell-free DNA in the urine for noninvasive clinical analysis. For example, cell-free DNA in the urine derived from blood cells can be used for noninvasive molecular analysis of hematological diseases and, cell-free DNA in urine derived from cells of the urinary tract may be used for noninvasive molecular analysis of any genetic diseases along the urinary tract. However, the sensitivity and clinical application of this new approach remain to be evaluated.

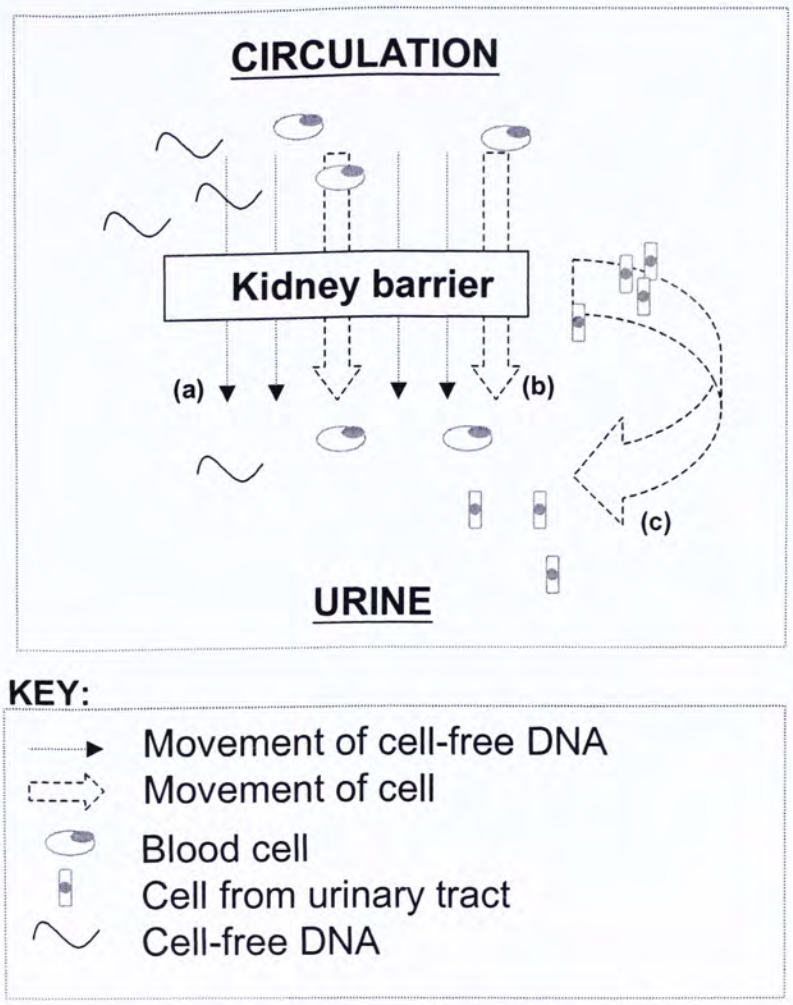


Figure 6.4 Schematic diagrams showing the three possible mechanisms resulting in the presence of cell-free DNA in the urine of HSCT recipients.

(a) represents the transrenal passage of cell-free DNA from the plasma into the urine. (b) represents the release of high-molecular weight cell-free DNA from blood cells liberated into the urine from the circulation. (c) represents the release of high-molecular weight cell-free DNA from cells liberated into the urine from the urinary tract.

SECTION V: CONCLUDING REMARKS

CHAPTER 7: CONCLUSION AND FUTURE PERSPECTIVES

7.1 Circulating miRNA is a valuable resource for molecular analysis

The discovery of placental mRNA transcripts in the plasma of pregnant women (Poon *et al.* 2000) has already opened up a new approach for noninvasive prenatal diagnosis (Ng *et al.* 2003a, Ng *et al.* 2003b). The recent discovery of miRNAs has prompted our team to investigate whether these small non-coding RNAs expressed in the placenta can also be detected in maternal plasma and be further developed as a novel class of circulating nucleic acids for molecular analysis. However, the existence and detection of miRNAs in human plasma has not been documented in the literature at the time of this writing.

In the work described in this thesis, I have developed and evaluated a new methodology for the extraction and quantitative detection of miRNA in maternal plasma (Chapter 3 and Chapter 4). Using this methodology, I have provided the first demonstration in the presence of circulating miRNAs in cell-free maternal plasma (Chapter 4). In addition, since this methodology was based on absolute, rather than relative quantification, I have also been able to determine that some miRNAs in plasma can reach as high as 10^7 copies per milliliter. These findings have revealed that miRNAs in plasma are stable enough for processing, extraction and can be robustly detected, and thus added a new and exciting dimension to the study of cell-free RNA species in plasma for noninvasive diagnosis and monitoring, not only for pregnancy, but also for other diseases and conditions.

After the confirmation in the existence of circulating miRNA, Chapter 5 has described a systematic search for the discovery of placental miRNAs in maternal

plasma. This strategy does not only allow the identification of placental miRNA markers without prior knowledge of the miRNAs being studied, but also provides quantitative information about the miRNA present in maternal plasma. In brief, using this strategy, Chapter 5 has reported a list of 17 candidate placental miRNAs potentially detectable in maternal plasma. In one of the placental miRNAs, miR-141, it was shown that it was present in maternal plasma of the first trimester pregnancies and the level of plasma miR-141 was shown to correlate with the gestational age of pregnancies. It implies the possibility of using miR-141 for clinical analysis in as early as the first trimester (12^{th} week) of pregnancy. These discoveries have opened up a completely new area of circulating pregnancy-specific miRNA markers for the potential applications in the diagnosis of pregnancy-associated disorders. Using this approach on other newly discovered miRNA, we should have a high chance of discovering other placental miRNAs for use in prenatal diagnosis.

It is interesting to note the number of similarities and differences between circulating miRNAs and mRNA transcripts. Firstly, the use of circulating mRNAs as biomarkers have been considered superior to the detection of circulating DNA in terms of its much larger copy numbers. This would allow a higher sensitivity of detection in maternal plasma. The surprisingly higher level of miRNAs, compared with that of mRNA, might provide an even more sensitive detection. Secondly, circulating mRNAs and miRNAs are both stably present in maternal plasma for extraction and robust quantification. However, unlike placental mRNA which is associated with subcellular particles, placental miRNA in maternal plasma is not associated with subcellular particles. The stability of miRNA over mRNA in maternal plasma is likely a result of its extremely short sequence as demonstrated by its long lasting existence in plasma after incubation. Purified and non-protected placental miRNA,

when incubated in plasma with nuclease activities, showed a slower rate of degradation and stayed at higher levels afer 2 hours of incubation than mRNA transcripts. Further investigation would be needed to see whether miRNA is associated with biochemical molecules, such as lipid, ribosome and protein. These studies will enhance understanding of both the mechanism and biological significance behind the release of nucleic acids into plasma.

In summary, this thesis has provided fundamental information and technical details on the potential application of a novel class of circulating nucleic acids, i.e. miRNA, in maternal plasma for noninvasive prenatal diagnostic tests. MiRNAs are stable and exist at very high concentration in plasma, enabling measurement with high precision. The biology and complex network of gene regulation by miRNAs also suggest the potential use of circulating miRNA for monitoring other diseases. For example, miRNA was found to be able to represent different cancer specifically. It is hopeful that future development in this field will enable a range of possible diagnostic tests based on circulating miRNAs.

7.2 The presence of donor-derived DNA in the urine of HSCT recipients

Apart from the cell-free nucleic acids in plasma, transrenal-DNA (Tr-DNA) in urine has recently attracted attention in its potential for noninvasive prenatal diagnosis. However, the detection of fetal DNA in urine cannot be reproduced by a number of groups (Zhong *et al.* 2001a, Li *et al.* 2003) and the phenomenon is in itself poorly understood. All these have hindered the development of Tr-DNA for noninvasive prenatal diagnosis. To address this problem, Chapter 6 of this thesis has tried to elucidate the constitutive components of cell-free DNA in urine using sex-mismatched HSCT patients and, thus, to illustrate the potential use of cell-free

DNA in urine, such as Tr-DNA, for noninvasive diagnostic tests. We hope the HSCT system may serve as an established model to stimulate further innovative researches on the phenomenon of Tr-DNA and cell-free DNA in urine in uses of prenatal diagnosis and cancer detection.

It is interesting to note that donor-derived DNA can be detected in the urine of these sex-mismatched HSCT patients. However, the data also suggested that cell-free DNA in the urine of HSCT patients had two possible sources: plasma cell-free DNA that has passed through the kidney barrier and high-molecular weight DNA that is released from the cellular portion of urine. The presence of high-molecular weight donor-derived and recipient-derived cell-free DNA in urine but not in plasma suggests the release of these DNA molecules from the cellular portion of urine. The correlation between the fractional concentrations of male DNA in urine and urine cell pellet has further suggested a dominant contribution of cell-free DNA in urine from cellular portion.

In brief, this study has raised important considerations regarding the detection of Tr-DNA and other cell-free DNA in urine for clinical analysis. For example, because the high-molecular weight DNA released from the urinary tract is an important component of cell-free DNA in urine, quantitative detection of Tr-DNA in urine should be designed in such a way to facilitate the detection of the smaller Tr-DNA fraction in the future. Moreover, the presence of high-molecular weight DNA released from cells of the urinary tract in urine hints on the use of these DNA for molecular analysis of disease conditions along the urinary tract. In summary, this part of the thesis has provided important information and technical details for the detection of cell-free DNA in urine. These cell-free DNA molecules included both

donor-derived DNA and recipient-derived DNA. They are both potentially enabling new perspective in the use of these cell-free DNA for noninvasive diagnostic tests in the future.

7.3 Prospects for future work

The detection of cell-free nucleic acids in human bodily fluids, such as plasma and urine, offer numerous opportunities for noninvasive diagnostic investigations. Over the past few years, encouraging findings have been reported on the detection and possible clinical applications of cell-free nucleic acids. Further development in this field will be encouraged by a better understanding of the physiological and pathological characteristics of these circulating cell-free miRNA species and cell-free DNA in urine.

In the first part of this thesis, I have investigated a systematic approach for identification of circulating placental miRNAs. Some of the molecular characteristics of these circulating miRNAs were also studied. One important future work is to investigate if the aberrant plasma levels of these placental-derived miRNAs can be observed in pathological pregnancies. For example, a recent study performed by Pineles *et al.* (2007) demonstrated that a distinct subset of miRNAs is differentially expressed in the placentas of pregnancies with pre-eclampsia and small-for-gestational age fetuses. It would be useful to investigate if the aberration of these miRNAs expressed in the pathological placentas would also be reflected in maternal plasma. Further experimentation might be performed to look for plasma miRNAs whose levels are altered in these pathological conditions. This would represent another step forward to developing circulating miRNAs as markers for noninvasive prenatal diagnosis. On the other hand, with more advanced molecular

techniques and high throughput technologies, such as miRNA microarray, the number of miRNAs discovered in humans would continue to increase. It is of interest to apply this approach, which requires no prior knowledge of the function of miRNAs, as described in Chapter 5 to identify more circulating placental miRNAs. Finally, because miRNAs are important regulators of gene expression, further investigations might be conducted to address the question of whether circulating miRNA possesses any functional implication in terms of feto-maternal communication.

In the latter part of this thesis, I have investigated in the constituting components of cell-free DNA in urine using HSCT patients to hint on the potential use of cell-free DNA in urine for diagnostic tests. It was found that the cell-free component consists of more than Tr-DNA. Conclusive evidence for presence of Tr-DNA requires further experimentation. In terms of future work, one might want to use techniques such as cell microscopy, flow cytometry and immunostaining to find out the cellular and genetic identities of cells in urine which contribute to majority of cell-free DNA in urine. Most importantly, after the establishment of the HSCT model for studying Tr-DNA, it would be worthwhile to study the different aspects of Tr-DNA, such as the mechanisms of formation and different biological and physical characteristic of Tr-DNA. It remains hopeful that the use of cell-free DNA in urine for molecular analysis would represent another important step in developing noninvasive diagnostic tests.

APPENDIX I

List of assay full names, target identities and sequences of TaqMan® MicroRNA Assays being employed in this thesis

AB Assay ID	miRNA Target	Target Sequence
abm000175	hsa-let-7a	ugagguaguagguuguauaguu
abm000176	hsa-let-7b	ugagguaguagguuguguggguu
abm000177	hsa-let-7d	agagguaguagguugcauagu
abm000178	hsa-let-7e	ugagguaggagguuguauagu
abm000179	hsa-let-7g	ugagguaguaguuuguacagu
abm000180	hsa-let-7i	ugagguaguaguuugugcu
abm000046	hsa-miR-100	aacccguagauccgaacuugug
abm000048	hsa-miR-103	agcagcauuguacagggcuauga
abm000049	hsa-miR-104	ucaacaucagucugauaagcua
abm000050	hsa-miR-105	ucaaaugcucagacuccugu
abm000051	hsa-miR-106a	aaaagugcuuacagugcagguagc
abm000053	hsa-miR-107	agcagcauuguacagggcuauca
abm000004	hsa-miR-10a	uacccuguagauccgaauuugug
abm000054	hsa-miR-122a	uggagugugacaaugguguuugu
abm000055	hsa-miR-124a	uuaaggcacgcggugaaugcca
abm000056	hsa-miR-124b	uuaaggcacgcggugaaugc
abm000057	hsa-miR-125a	ucccugagacccuuuaaccugug
abm000058	hsa-miR-125b	ucccugagacccuaacuuguga
abm000059	hsa-miR-126	ucguaccgugaguaauaaugc
abm000060	hsa-miR-127	ucggauccgucugagcuuggcu
abm000061	hsa-miR-128a	ucacagugaaccggucucuuuu
abm000062	hsa-miR-128b	ucacagugaaccggucucuuuc
abm000063	hsa-miR-129	cuuuuugcggucugggcuugc
abm000064	hsa-miR-130a	cagugcaauguuaaaagggc
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abm000071	hsa-miR-135b	uauggcuuuucauuccuaugug
abm000073	hsa-miR-137	uauugcuuaagaauacgcguag
abm000074	hsa-miR-138	agcuggugguugaauc
abm000075	hsa-miR-139	ucuacagugcacgugucu
abm000076	hsa-miR-140	agugguuuuacccuaugguag
abm000077	hsa-miR-141	aacacugucuggguaaagaugg
abm000078	hsa-miR-142-3p	uguaguguuuccuacuuuaugga
abm000079	hsa-miR-142-5p	cauaaaguagaaagcacuac
abm000081	hsa-miR-144	uacaguauagaugauguacuag
abm000082	hsa-miR-145	guccaguuuucccaggaaucccuu

abm000083	hsa-miR-146	ugagaacugaauuccauggguu
abm000084	hsa-miR-147	guguguggaaaugcuucugc
abm000085	hsa-miR-148a	ucagugcacuacagaacuuugu
abm000087	hsa-miR-149	ucuggcuccgugucuucacucc
abm000088	hsa-miR-150	ucucccaacccuuguaccagug
abm000089	hsa-miR-151	acuagacugaagcuccuugagg
abm000090	hsa-miR-152	ucagugcaugacagaacuugg
abm000092	hsa-miR-154	uagguuauccguguugccuucg
abm000093	hsa-miR-154*	aaucauacacgguugaccuauu
abm000094	hsa-miR-155	uuaaugcuaaucgugauagggg
abm000006	hsa-miR-15a	uagcagcacauaaugguuugug
abm000007	hsa-miR-15b	uagcagcacaucaugguuuaca
abm000008	hsa-miR-16	uagcagcacguaaauauuggcg
abm000009	hsa-miR-17-3p	acugcagugaaggcacuugu
abm000010	hsa-miR-17-5p	caaagugcuuacagugcagguagu
abm000095	hsa-miR-181a	aacauucaacgcugucggugagu
abm000096	hsa-miR-181b	aacauucauugcugucgguggguu
abm000097	hsa-miR-181c	aacauucaaccugucggugagu
abm000098	hsa-miR-182	uuuggcaaugguagaacucaca
abm000099	hsa-miR-182*	ugguucuagacuugccaacua
abm000100	hsa-miR-183	uauggcacugguagaauucacug
abm000101	hsa-miR-184	uggacggagaacugauaagggu
abm000102	hsa-miR-185	uggagagaaaggcaguuc
abm000103	hsa-miR-186	caaagaauucuccuuuugggcuu
abm000104	hsa-miR-187	ucgugucuuguguugcagccg
abm000106	hsa-miR-189	gugccuacugagcugauaucagu
abm000107	hsa-miR-190	ugauauguuugauauauuaggu
abm000108	hsa-miR-191	caacggaaucccaaaagcagcu
abm000109	hsa-miR-193	aacuggccuacaaagucccag
abm000110	hsa-miR-194	uguaacagcaacuccaugugga
abm000111	hsa-miR-195	uagcagcacagaaauauuggc
abm000112	hsa-miR-197	uucaccaccuucuccacccagc
abm000113	hsa-miR-198	gguccagaggggagauagg
abm000114	hsa-miR-199a	cccaguguucagacuaccuguuc
abm000115	hsa-miR-199a*	uacaguagucugcacauugguu
abm000116	hsa-miR-199b	cccaguguuuagacuaucuguuc
abm000117	hsa-miR-199-s	cccaguguucagacuaccuguu
abm000011	hsa-miR-19a	ugugcaaaucuaugcaaaacuga
abm000013	hsa-miR-20	uaaagugcuuauagugcaggua
abm000118	hsa-miR-200a	uaacacugucugguaacgaugu
abm000119	hsa-miR-200b	cucuaauacugccugguaaugaug
abm000120	hsa-miR-200c	aauacugccggguaaugaugga
abm000121	hsa-miR-203	gugaaauguuuaggaccacuag
abm000122	hsa-miR-204	uucccuuugucauccuaugccu
abm000123	hsa-miR-205	uccuucauuccaccggagucug
abm000014	hsa-miR-21	uagcuuaucagacugauguuga

abm000126	hsa-miR-210	cugugcgugugacagcggcug
abm000127	hsa-miR-211	uucccuuugucauccuucgccu
abm000128	hsa-miR-213	accaucgaccguugauuguacc
abm000129	hsa-miR-214	acagcaggcacagacaggcag
abm000130	hsa-miR-215	augaccuaugaauugacagac
abm000131	hsa-miR-216	uaaucucagcuggcaacugug
abm000133	hsa-miR-218	uugugcuugaucuaaccaugu
abm000134	hsa-miR-219	ugauuguccaaacgcaauucu
abm000135	hsa-miR-220	ccacaccguaucugacacuuu
abm000136	hsa-miR-221	agcuacauugucugcuggguuuc
abm000137	hsa-miR-222	agcuacaucuggcuacugggucuc
abm000138	hsa-miR-223	ugucaguuugucaaauacccc
abm000139	hsa-miR-224	caagucacuagugguuccguuua
abm000016	hsa-miR-23a	aucacauugccagggauuucc
abm000017	hsa-miR-23b	aucacauugccagggauuaccac
abm000019	hsa-miR-25	cauugcacuugucucggucuga
abm000020	hsa-miR-26a	uucaaguaauccaggauaggcu
abm000021	hsa-miR-26b	uucaaguaauucaggauaggu
abm000022	hsa-miR-27a	uucacaguggcuaaguuccgcc
abm000023	hsa-miR-27b	uucacaguggcuaaguucug
abm000024	hsa-miR-28	aaggagcucacagucuauugag
abm000140	hsa-miR-296	agggccccccucaauccugu
abm000141	hsa-miR-299	ugguuuaccgucccacauacau
abm000025	hsa-miR-29a	cuagcaccaucugaaaucgguu
abm000026	hsa-miR-29b	uagcaccauuugaaaucagu
abm000027	hsa-miR-29c	uagcaccauuugaaaucgguua
abm000142	hsa-miR-301	cagugcaauaguauugucaaagc
abm000143	hsa-miR-302a	uaagugcuuccauguuuugguga
abm000144	hsa-miR-302b	uaagugcuuccauguuuuaguag
abm000145	hsa-miR-302b*	acuuuaacauggaagugcuuucu
abm000146	hsa-miR-302c	uaagugcuuccauguuucagugg
abm000147	hsa-miR-302c*	uuuaacaugggguaccugcug
abm000148	hsa-miR-302d	uaagugcuuccauguuugagugu
abm000028	hsa-miR-30a-3p	cuuucagucggauguuugcagc
abm000029	hsa-miR-30b	uguaaacauccuacacucagc
abm000030	hsa-miR-30c	uguaaacauccuacacucucagc
abm000031	hsa-miR-30d	uguaaacaucccgacuggaag
abm000032	hsa-miR-30e	uguaaacauccuugacugga
abm000033	hsa-miR-31	ggcaagaugcuggcauagcug
abm000149	hsa-miR-320	aaaagcuggguugagagggcgaa
abm000151	hsa-miR-323	gcacauuacacggucgaccucu
abm000153	hsa-miR-324-5p	cgcauccccuagggcauuggugu
abm000154	hsa-miR-325	ccuaguagguguccaguaagu
abm000155	hsa-miR-326	ccucugggcccuuccuccag
abm000156	hsa-miR-328	cuggcccucucugcccuuccgu
abm000157	hsa-miR-330	gcaaagcacacggccugcagaga

abm000158	hsa-miR-331	gccccugggccuauccuagaa
abm000159	hsa-miR-335	ucaagagcaauaacgaaaaaugu
abm000160	hsa-miR-337	uccagcuccuauaugaugccuuu
abm000161	hsa-miR-338	uccagcaucagugauuuuguuga
abm000162	hsa-miR-339	ucccuguccuccaggagcuca
abm000163	hsa-miR-340	uccgucucaguuacuuuauagcc
abm000164	hsa-miR-342	ucucacacagaaaucgcacccguc
abm000036	hsa-miR-34a	uggcagugucuuagcugguugu
abm000037	hsa-miR-34b	aggcagugucauuagcugauug
abm000038	hsa-miR-34c	aggcaguguaguuagcugauug
abm000166	hsa-miR-367	aauugcacuuuagcaaugguga
abm000167	hsa-miR-368	acauagaggaaauuccacguuu
abm000169	hsa-miR-370	gccugcugggguggaaccugg
abm000170	hsa-miR-371	gugccgccaucuuuugagugu
abm000171	hsa-miR-372	aaagugcugcgacauuugagcgu
abm000172	hsa-miR-373	gaagugcuucgauuuuggggugu
abm000173	hsa-miR-373*	acucaaaaugggggcgcuuucc
abm000174	hsa-miR-374	uuauaauacaaccugauaagug
abm001112	hsa-miR-515-5p	uucuccaaaagaaagcacuuucug
abm001157	hsa-miR-518c	caaagcgcuucucuuuagagug
abm001169	hsa-miR-520d	ucuacaaagggaagcccuuucug
abm001175	hsa-miR-526b	cucuugagggaagcacuuucuguu
abm000002	hsa-miR-9	ucuuugguuaucuagcuguauga
abm000003	hsa-miR-9*	uaaagcuagauaaccgaaagu
abm000039	hsa-miR-92	uauugcacuugucccggccugu
abm000041	hsa-miR-95	uucaacggguauuuauugagca
abm000042	hsa-miR-96	uuuggcacuagcacauuuuugc
abm000043	hsa-miR-98	ugagguaguaaguuguauuguu
abm000044	hsa-miR-99a	aacccguagauccgaucuugug

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